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Rheumatoid arthritis and periodontitis: antibody response, oral  
microbiome, cytokine profile and effect of periodontal treatment

By

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DOCTOR OF PHILOSOPHY

School of Dentistry  
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## Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects about 1% of the world population. This common disease is characterized by chronic inflammation of the synovium that leads to destruction of cartilage and bone in the joint, and the cause of this exacerbated inflammatory reaction remains unknown. Periodontitis (PD) is also a chronic inflammatory disease characterized by destruction of bone and other connective tissue that shares notable similarities with RA. Over the last 20 years, numerous studies have found an epidemiological connection between RA and periodontitis. However the biological mechanisms that explain the interrelations between the two conditions are not known. The main hypothesis sustains that periodontal bacteria (mainly *Porphyromonas gingivalis*) could have a role in the progression of RA. Also, recent investigations suggest that periodontal therapy could reduce inflammation in RA, but the results are not conclusive.

The aim of this thesis was to investigate the role of periodontitis in RA and the effect of periodontal therapy on immunological and microbiological parameters. To do that, different biological samples were collected from two pilot studies, comparing RA and periodontitis patients to the appropriate controls (i.e. RA periodontally healthy, no RA periodontitis and no RA no periodontitis) and from a selected group of randomized RAPD patients before and after periodontal therapy. The antibody response and subgingival microbiome of patients with RA and periodontitis were compared to the appropriate controls (no RA no PD, RA no PD, no RA PD). The effect of periodontal therapy on these parameters and on the cytokine changes in gingival crevicular fluid was also investigated.

The findings from this thesis lend further credence to the link between RA and the oral microbiome, with RA patients having a disrupted and more anaerobic microflora and an exacerbated immunological reaction against periodontal bacteria and citrullinated proteins

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## Abbreviations

AAG: Alpha-1 Acid Glycoprotein

ACPA: Anti-Citrullinated Protein Antibody

APC: Antigen Presenting Cell

BoP: Bleeding on Probing

BSA: Bovine Serum Albumin

CAL: Clinical Attachment Loss

CEP: Citrullinated human alpha-enolase

CCP: Cyclic Citrullinated Peptide

CPD, cumPD: Cumulative Probing Depth

CRP: C-Reactive Protein

DAS: Disease Activity Score

ELISA: Enzyme-Linked Immuno-Sorbent Assay

EULAR: European League Against Rheumatism

ESR: Erythrocyte Sedimentation Rate

Fib: Fibrinogen

FN: *Fusobacterium nucleatum*

GI: Gingival Index

GMCSF: Granulocyte-Macrophage Colony-Stimulator growth Factor

HAQ: Health Assessment Questionnaire

IL: Interleukine

IQR: Interquartile Range

N, n: Number of patients

NETs: Neutrophil Extracellular Traps

OR: Odds Ratio

OTU: operational taxonomic unit

PBS: Phosphate-buffered Saline

PI: plaque index

PD: periodontitis;

*P.gingivalis*, PG: *Porphyromonas gingivalis*

PAD: Peptidyl Arginine Deiminase

PPAD: Peptidyl Arginine Deiminase from *P.gingivalis*

RA: Rheumatoid Arthritis

RR: Risk Ratio

RF: Rheumatoid Factor

SD: Standard Deviation

SEM: Standard Error of the Mean

TNC-5: Tenascin 5

TNF: Tumor Necrosis Factor

VAS: Visual Analogue Scale

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# 1. INTRODUCTION

### 1.1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease that affects about 1% of the world population, being more prevalent in women and in the 4th and 5th decade of life (Sacks, Luo et al. 2010). It is believed that the prevalence of RA in the first world is increasing, and in the UK 26,000 patients are being diagnosed every year (Garcia Rodriguez, Tolosa et al. 2008), (Cross, Smith et al. 2014). While the cause of RA remains unknown, the pathogenesis is characterized as chronic inflammation of the synovium that leads to destruction of cartilage and bone (Feldmann, Brennan et al. 1996). This constant state of inflammation and degradation of the joint structures causes deformations, functional impairment and disability accompanied by chronic pain and constitutional symptoms. In addition, the mortality of RA patients is increased due to comorbidities associated with RA such as cardiovascular disease (Wållberg-Jonsson, Johansson et al. 1999).

The understanding of the pathogenesis of RA has increased over the last decade, with the study of the role of inflammatory cells such as macrophages, B cells and CD4+T cells, and pro-inflammatory cytokines and degradative enzymes, which are elevated in the RA synovium and are capable of digesting the extracellular matrix, causing joint destruction. The inflammatory process that occurs in the synovium of RA patients produces pro-inflammatory cytokines including Tumor necrosis factor (TNF)-alpha, Interleukins (IL) 1, 6, 15, 18, granulocyte-macrophage colony-stimulating factor (GM-CSF). In the presence of these cytokines, T-cells and synoviocytes activate osteoclast maturation, which leads

to bone degradation. In addition to the inflammation of the synovium, the pannus (an anomalous fibrovascular coating) invades and destroys the joint surface (Firestein 2003), (Niu and Chen 2014).

The typical signs and symptoms of RA are general pain and swelling of joints (often symmetrically), morning stiffness and limitation to movement that last more than one hour and that can be reduced by gentle movements. These clinical signs help the differential diagnosis with osteoarthritis (OA), which is not normally presented with swelling, the morning stiffness lasts less than an hour and the joint movement can worsen the pain instead of helping it, as OA is caused by cartilage degeneration and mechanical wear (Ropes, Bennett et al. 1957).

The 2010 American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) devised new classification criteria, which uses a score-based algorithm considering type and number of joints involved, serology parameters (Rheumatoid Factor and Anti Citrullinated Protein Antibody results), acute-phase reactants (C-reactive protein and Erythrocyte Sedimentation Rate) and duration of the symptoms. A score of 6 out of 10 is needed to diagnose the patient with definitive RA (Aletaha, Neogi et al. 2010). One of the new diagnostic tools of this classification criteria are the presence of antibodies against citrullinated proteins (ACPA), which are found in 80% of RA patients and have approximately 99% specificity, being the most disease-specific antibody in Rheumatoid Arthritis, appearing even before clinical signs (Van Gestel, Anderson et al. 1999).

The pharmacological treatment of RA aims to relieve pain, reduce inflammation and prevent destruction of cartilage and bone. Rheumatologists often use a number of different drugs to achieve this, such as non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids (most often by injection) and conventional disease-modifying anti-rheumatic drugs (DMARDs). This last group of drugs acts on the immune system to retard the inflammatory progression of RA. Biological therapies, a subcategory of DMARDs, target specific steps of the inflammatory process and have been proved to be very effective in patients with active RA that have not responded to other treatment. A very common biological therapy, anti TNF, in combination with conventional DMARDs provides clinical benefits and halts the progression of joint damage (Lipsky, van der Heijde et al. 2000).

## 1.2. Chronic periodontitis

Periodontitis is a disease characterized by destruction of the connective tissues supporting the teeth. Chronic periodontitis one of the most prevalent diseases affecting humans and, according to the last European Workshop on Periodontology, 11% of the adult population is affected by severe periodontitis (Papapanou and Susin 2017). However, the prevalence of chronic periodontitis (often referred to as periodontitis) is difficult to estimate due to the lack of consensus in the definition and diagnosis of the disease (Holtfreter, Albandar et al. 2015).

Chronic periodontitis is characterized by the presence of gingival inflammation that leads to destruction of the periodontal ligament. This state of chronic inflammation also results in resorption of the alveolar bone supporting the teeth (Armitage 1995), being a major cause of tooth loss in adults, especially in the elderly (Phipps and Stevens 1995). Although it often appears as asymptomatic, clinical signs like swollen and tender gums, bleeding after brushing or spontaneously, recession of the gums, increased space between teeth, pathological movement of teeth, bad taste and halitosis are common in periodontitis patients (Armitage 1995).

Some of these symptoms also appear in gingivitis, which precedes periodontitis but, in gingivitis, the inflammation of the gums can be reversible as there is no loss of alveolar bone. To clinically distinguish between gingivitis and periodontitis,

it is necessary to determine whether there has been loss of the supporting tissues of the teeth. Although there is no consensus on the clinical definition of periodontitis, the diagnosis is based on the radiographic bone loss and the clinical evaluation of the loss of periodontal tissue using a periodontal probe to measure the clinical attachment loss (CAL) and probing depth (PD) (Armitage 1995) (Savage, Eaton et al. 2009).

The pathogenesis of periodontal disease is initiated by periodontal bacteria but propagated by the destructive response of the host immune system. Periodontal pathogens are mainly Gram-negative anaerobes and spirochetes. The main organisms that have been associated with periodontitis are *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Treponema denticola*, and *Porphyromonas gingivalis* (Socransky, Haffajee et al. 1998). These microorganisms produce enzymes and toxins that damage the periodontal tissue and activate an inflammatory immune response releasing chemotactic peptides that attract immune cells to the site of infection (Kinane 2001). The activated immune cells and damaged epithelial cells generate chemotactic signal molecules (interleukin-8, complement C5a, etc) to recruit neutrophils. As a collateral effect, neutrophils release elastase, matrix metalloproteinases and reactive oxygen species causing significant tissue damage (Cooper, Palmer et al. 2013). These cytokines released, especially IL-1 and IL-6 activate the expression of receptor activator of nuclear factor-kappa B ligand (RANKL) in osteoblasts, which binds to RANK from osteoclasts activating their differentiation and survival. Breaking the ratio between RANKL and its decoy receptor OPG

(Osteoprotegerin) results in bone resorption (Lu, Chen et al. 2006). In response to this bone loss, the epithelial cells migrate apically and the periodontal pocket becomes deeper, facilitating the accumulation of subgingival plaque, the survival of anaerobic pathogens and disease progression (Phipps and Stevens 1995).

The aim of periodontal therapy is to restore the pro-inflammatory state of the gingival tissues by reducing the pathogenic subgingival-microflora. This consists of manual and/or ultrasonic sub-gingival debridement and scaling and root planing (Cobb 2002). Some researchers recommend the local use of chlorhexidine as an adjunct to periodontal therapy (Soskolne, Heasman et al. 1997) (Greenstein, Berman et al. 1986). In general, effective sub-gingival scaling improves the clinical parameters such as BoP, probing depth and CAL and it also shifts the periodontal microflora by reducing the load of Gram-negative microbes which allows an increase in the Gram-positive microbes associated with gingival health (Takamatsu, Yano et al. 1999) (Hinrichs, Wolff et al. 1985). In general, the consensus regarding the criteria for success used for periodontal treatment is to stop further clinical attachment loss and to restore the clinical parameters to health. This includes achieving a probing depth lower than 4mm, no BoP, no mobility, etc. (Lindhe, Westfelt et al. 1984), (Yaghini, Tavakoli et al. 2011). However, the effectiveness of the treatment decreases in deep pockets, especially when probing depths exceed 5 mm (Cobb 1996). Residual pockets of more than 4 mm post-treatment have been associated with the presence of *P. gingivalis* (Mombelli, Schmid et al. 2000).

### 1.3. Epidemiological association between Periodontitis and RA



The relationship between periodontitis and systemic diseases has become of great interest in the last twenty years. Periodontitis has been strongly associated with the development of diabetes (Preshaw, Alba et al. 2012) (Chapple and Genco 2013) and cardiovascular disease (Friedewald, Kornman et al. 2009) (Haraszthy, Zambon et al. 2000). Furthermore, there is evidence of association between periodontitis and adverse pregnancy outcomes and with other systemic diseases such as COPD or rheumatoid arthritis (Hajishengallis 2015).

Over the last 20 years, numerous studies have identified an association between periodontitis and tooth loss and rheumatoid arthritis (Table 1. 1). While two of these studies reported negative results, the majority observed a higher risk and prevalence of periodontitis in rheumatoid arthritis patients and vice versa.

In cross sectional studies, researchers found that, patients with RA had a significantly increased prevalence of periodontitis compared to systemically healthy controls, with odds ratios (OR) ranging between 1.82 (de Pablo, Dietrich et al. 2008) and 8.1 (Pischon, Pischon et al. 2008). Furthermore, patients with periodontitis have higher prevalence of RA, with OR ranging between 1.16 (Potikuri, Dannana et al. 2012) to 2.05 (Demmer, Molitor et al. 2011). Some of these studies, after adjusting for confounding factors, showed that this relationship is independent of smoking (Potikuri, Dannana et al. 2012), oral hygiene (plaque) (Mercado, Marshall et al. 2001), (Pischon, Pischon et al. 2008) and genetic factors (Marotte, Farge et al. 2006).

In the National Health and Nutrition Examination Survey (NHANES) data reported by De Pablo et al., 50% of RA patients were identified as edentulous, with these patients maybe representing severe cases of periodontitis (de Pablo, Dietrich et al. 2008). Similarly, in one of the largest cohort studies investigating the Taiwanese National Health Insurance Research Database (NHIRD), authors found a significant and independent association between RA and history of periodontitis (Chen, Huang et al. 2013). Another study reported that from the 74 RA patients examined, 94% suffered from moderate to severe periodontitis (48% moderate and 46% severe) (Monsarrat, Vergnes et al. 2014).

However, there are also studies that did not find such associations. In a large cohort study, using data from 91,132 nurses followed over 12 years, there was no evidence of a higher risk of developing RA patients among those with periodontitis; however, periodontitis was defined based on history of periodontal surgery and therefore included individuals with treated and not active periodontitis (Arkema, Karlson et al. 2010). More recently, no difference in the prevalence of periodontitis was observed in a Swedish case-control study including 2,740 RA patients in whom periodontitis was defined by self-report (Eriksson, Nise et al. 2016).

The most recent systematic review with meta-analysis considering the epidemiological evidence from 8 case control studies, concluded that there is a strong and significant association between RA and to PD. However, the authors

highlight the need of rigorous studies using consistent definition of periodontitis and a more defined population to avoid bias due to invalid control groups (Tang, Fu et al. 2017).

Table 1. 1 Summary of observational studies published since 2000 (with study population>60) studying the association between rheumatoid arthritis (RA) and periodontitis. Abbreviations: OR, odds ratio; RR, risk ratio; PI, plaque index; GI, gingival index; RF, rheumatoid factor; anti-CCP, anti-cyclic Citrullinated Peptide.

Author & Year	Study population	Results	Type of study
(Mercado, Marshall et al. 2000)	1412 patients attending dental hospital	Periodontitis: higher prevalence of RA (3.95%) vs no RA	Cross sectional
(Mercado, Marshall et al. 2001)	65 patients (RA vs. non-RA)	-RA: higher number of missing teeth, deeper pockets. No difference in bleeding or plaque index	Case control
(Marotte, Farge et al. 2006)	147 patients (RA)	-Association between periodontal bone loss and wrist bone destruction ( $\chi^2=11.82$ ) and shared epitope HLA-DR	Cross sectional
(de Pablo, Dietrich et al. 2008)	4,461 patients (NHANES III)	-RA: higher prevalence of periodontitis (OR:1.82), edentulous (OR:2.27), less decay ( $p<0.001$ )	Cross sectional
(Pischon, Pischon et al. 2008)	109 patients (57 RA, 52 non-RA)	RA: higher prevalence of periodontitis (OR:8.05), statistically significant after adjusting to confounding factors (PII, GI)	Case control
(Dissick, Redman et al. 2009)	69 RA patients vs. 35 Osteoarthritis (controls) patients	-RA: higher prevalence of periodontitis and more severe -RA patients with periodontitis associated with RF positive and anti-CCP	Case control

(Arkema, Karlson et al. 2010)	81,132 patients (Nurses' Health Study prospective cohort)	No evidence of higher incidence of RA in periodontitis	Cohort study
(Demmer, Molitor et al. 2011)	9,702 patients (NHANNES I)	Periodontitis: higher prevalence of RA (OR:2.05)	Cross sectional
(Potikuri, Dannana et al. 2012)	91 RA (DMARD naive, non-smokers) vs healthy controls	RA: higher prevalence of periodontitis (OR:4.28)	Case control
(Smit, Westra et al. 2012)	95 RA, 420 matched controls	RA: higher risk of periodontitis (RR: 3.7)	Cross sectional
(Chen, Huang et al. 2013)	13,779 newly diagnosed RA, 137,790 non-RA	Periodontitis: higher prevalence of RA (OR:1.16) ,	Cohort study
(Monsarrat, Vergnes et al. 2014)	74 RA patients	94% of RA had periodontitis (48% moderate and 46% severe)	Cross sectional
(Eriksson, Nise et al. 2016)	2,740 RA cases and 3,942 non-RA	No difference in periodontitis prevalence between groups	Case control

#### 1.4. Outcomes of periodontal treatment in RA

For a long time, researchers have believed that treating oral health problems improved rheumatoid arthritis. The first records date to the time of Hippocrates, who suggested that, the cure of arthritis included tooth extraction. Many years later, in the 17th century, Benjamin Rush proposed that rheumatism could be cured by removal of infected teeth. During the 1950s-60s, “total dental clearance” was used for treatment of RA (Rothschild 2017).

A few small clinical studies evaluating the effect of non-surgical periodontal therapy on RA suggest that treatment of periodontitis may have a significant positive effect on RA severity (Table 1. 2) (Ribeiro, Leao et al. 2005) (Ortiz, Bissada et al. 2009) (Okada, Kobayashi et al. 2013) (Erciyas, Sezer et al. 2013). Due to its high prevalence, periodontitis may represent an important modifiable factor for RA incidence and severity. If proved, treatment of periodontitis could be an inexpensive and safe, non-pharmacological treatment with direct benefit for patients with RA. Although results are promising, some authors failed to see this effect (Zhang and Zhang 2010). In a randomized clinical trial, Pinho and collaborators did not observe a reduction in acute phase reactants after periodontal therapy in RA and these systemic markers of inflammation were not correlated with periodontal improvement (Pinho, Oliveira et al. 2009).

To date, only a few randomized clinical trials have investigated the effect of periodontal therapy on RA and these studies have a short follow-up period

(ranging between 8 weeks and 6 months) and a small sample size (<75 patients). Furthermore, each study uses different definition of periodontitis and uses different parameters to measure RA status, being the most used the Disease Activity Score known as DAS28, based on subjective measures like the Visual Analog Scale (VAS) and number of tender joints. Due to the heterogeneity in all the studies connecting RA and periodontitis, in the systematic review completed by Kaur et al., only a few parameters could be included for meta-analysis; ESR being the only test found to be significantly reduced after periodontal treatment (Kaur, Bright et al. 2014). One of the most recent studies found a significant reduction in DAS28, ESR and CRP in 30 RAPD patients undergoing periodontal therapy, compared to controls (Khare N 2016). Therefore, researchers in the field agree that there is a gap in the scientific literature as more long controlled randomized controlled trials are needed to demonstrate an outcome in RA through periodontal treatment.

In the workshop between the European Federation of Periodontology and the American Academy of Periodontology in 2013, it was concluded that more rigorous clinical controlled trials and research was needed in the field (Linden and Herzberg 2013).

Table 1. 2 Clinical trials evaluating the effect of periodontal therapy in Rheumatoid arthritis (RA). Abbreviations: PD, periodontitis; RA, Rheumatoid Arthritis; RF, Rheumatoid Factor, ESR, Erythrocyte Sedimentation Rate; HAQ, Health Assessment Questionnaire; DAS, Disease Activity Score; VAS, Visual Analogue Scale; TNF, Tumor Necrosis Factor; CRP, C-Reactive Proteins; AAG, alpha-1 acid glycoprotein.

Author& year	Duration	Patient number	Parameters evaluated	Results
Ribeiro 2005(Ribeiro, Leao et al. 2005)	3 months	42 RA+PD -16 periodontal treatment -26 oral hygiene and supragingival cleaning	RF, ESR, HAQ	RF significantly reduced
Al-katma 2007(Al-Katma, Bissada et al. 2007)	8 weeks	29 RA+PD -17 periodontal treatment -12 no treatment	DAS 28, ESR	VAS, DAS and ESR reduced
Khare 2016 (Khare N 2016)	3 months	60 RAPD -30 periodontal treatment -30 no treatment	OHI, BoP, PPD, CAL, SJ, TJ, ESR, VAS, DAS28, CRP	ESR, DAS28, VAS, CRP significantly reduced
Ortiz 2009(Ortiz, Bissada et al. 2009)	8 weeks	40 RA+PD -10 periodontal treatment and DMARDs only. -10 periodontal treatments and DMARDs with anti-TNF drugs. -10 no periodontal therapy, DMARDs only -10 no periodontal treatment, DMARDs with anti-TNF drugs.	ESR, TNF-alpha, signs and symptoms	VAS and DAS improved in treatment groups. ESR not significantly reduced Anti-TNF drugs improved PPD and CAL
Pinho 2009(Pinho, Oliveira et al. 2009)	6 months	75 patients: -15 RA+PD with periodontal treatment -15 RA+PD no periodontal treatment	DAS 28, CRP, ESR, AAG (alpha-1 acid glycoprotein)	No clear relation. AAG, ESR and CRP not significantly reduced with



		-15 PD with periodontal treatment -15 PD no periodontal treatment		periodontal therapy.
Okada 2013(Okada, Kobayashi et al. 2013)	8 weeks	55 RA+PD -26 supragingival cleaning -29 no treatment	DAS 28, CRP, anti-CCP, RF, TNF-alpha and levels of IgG to <i>P. gingivalis</i>	Reduction of DAS 28 and levels of IgG to <i>P. gingivalis</i> and citrulline.
Erciyas 2013(Erciyas, Sezer et al. 2013)	3 months	60 RA and PD -30 RA moderate-severe disease activity -30 RA low disease activity	ESR, CRP, TNF-alpha, DAS28	Significant reduction of ESR, CRP, TNF-alpha, DAS28

### 1.5. Possible biological mechanisms:

The notable similarities between RA and periodontitis have been object of study for many years (Bingham III and Moni 2013). However, it is still unclear the biological mechanisms that explain the interrelations between the two conditions and different possible theories have been described:

#### 1.5.1. Shared risk factors

The HLA-DR4 epitope (human leukocyte antigen, alleles DR4) is located in the surface of leukocytes and has been found to be associated with both RA and periodontitis (Gregersen, Silver et al. 1987). Numerous studies have reported an association between periodontitis and the HLA gene. The most recent systematic review reported a protective association with HLA-A2 and B5 and a susceptibility factor for periodontal disease with HLA-A9 and B15 (Stein, Machulla et al. 2008). However, the HLA associations alone are not enough to explain the link between RA and PD (Marotte, Farge et al. 2006) (Potikuri, Dannana et al. 2012).

Oral hygiene has also been proposed to be the link between rheumatoid arthritis and periodontitis. A common misconception is that RA patients have impaired dexterity and accordingly likely to have poorer oral hygiene than healthy control subjects. Although, due to the joint and bone deformities typically affecting the hands of RA patients, it seems logical that these patients would have an impediment to maintain a good oral hygiene, studies have consistently failed to show a difference in plaque control that could explain the association with

periodontitis (Torkzaban, Hjiabadi et al. 2012) (Mercado, Marshall et al. 2001) (Pischon, Pischon et al. 2008).

Smoking is also a shared risk factor for RA and periodontitis. RA patients have higher prevalence of PD but also patients with new-onset RA exhibit a high prevalence of PD at disease onset, despite their young age and low smoking history (Scher, Ubeda et al. 2012). Although periodontitis and RA share genetic (HLD-DR4) and risk factors (smoking) and similar inflammatory pathways, it is not believed to be enough to explain this connection (Kaur, White et al. 2013).

#### 1.5.2. Citrullination

Citrullination or deimination is a post-translational modification in which the amino acid arginine is transformed into citrulline, catalysed by the enzyme Peptidyle-Arginine Deiminase (PAD). In the presence of calcium, PAD substitutes the ketamine group ( $=NH$ ) with a ketone group ( $=O$ ), converting arginine into citrulline and neutralising the previous positive charge of the amino acid (Baka, György et al. 2012) (Figure 1. 1).

Citrullination occurs as a physiological phenomenon in healthy individuals and it is essential for skin physiology, gene regulation and correct functioning of the immune system (Baka, György et al. 2012). However, it has also been implicated in autoimmune diseases such as psoriasis and rheumatoid arthritis.



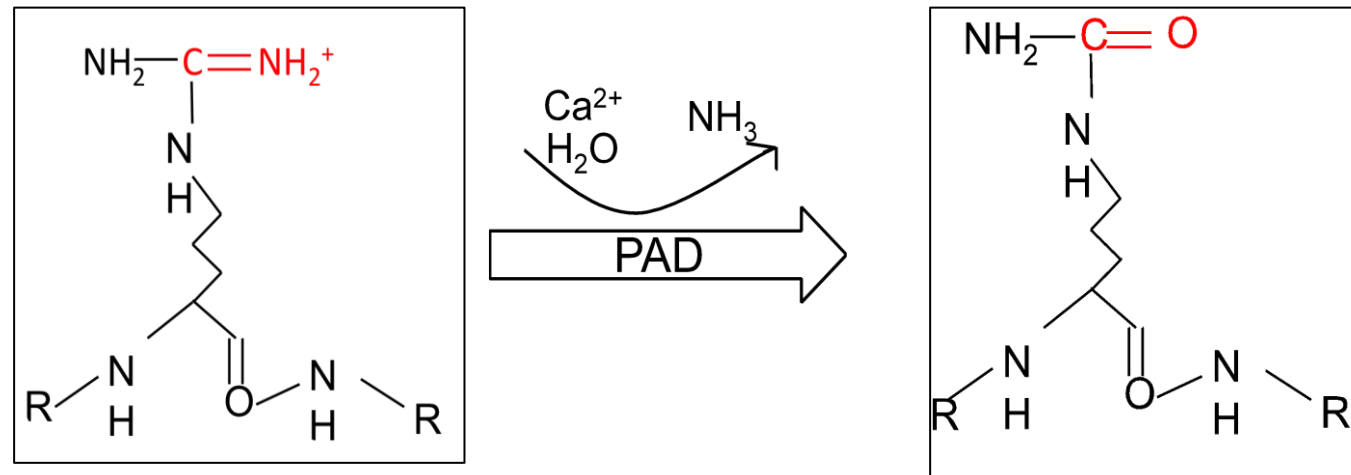


Figure 1. 1 Protein citrullination by Peptidyl-Arginine Deiminase (PAD). In the presence of calcium, PAD catalyses the conversion of Arginine in to citrulline, changing the ketamine group ( $=\text{NH}$ ) for a ketone group ( $=\text{O}$ ).

In rheumatoid arthritis the expression of PAD 4 and PAD 2 in synovium is correlated with intensity of inflammation (Foulquier, Sebbag et al. 2007). These enzymes, during oxidative stress or apoptosis, become active and citrullinate proteins.

While elevation of citrullination is relatively common in other diseases, the production of auto-antibodies against these epitopes, is rather specific to RA. ACPAs are found in 80% of patients with RA and are synthesized by plasma cells to target citrullyl residues. These auto-antibodies are highly specific for RA (98%) and have been found in serum of patients years before they develop any clinical signs, being the earliest and most specific autoantibodies in RA (Schellekens, Visser et al. 2000), (Nielen, van Schaardenburg et al. 2004). This unique production of auto-antibodies in RA suggest the necessity of an external factor to trigger this auto-immune reaction.

#### 1.5.1. *Porphyromonas gingivalis* citrullination

*Porphyromonas gingivalis* (PG) is one of the bacteria associated with periodontitis and is considered key to the disruption of the host-microbial homeostasis that causes this disease (Darveau, Hajishengallis et al. 2012). Among the variety of virulence factors that PG uses for tissue destruction and host evasion, this Gram-negative anaerobe has a family of enzymes called gingipains, which not only helps the bacteria in colonization and nutrition but it can neutralize the host defenses and manipulate the inflammatory response (Amano 2003).

However, the most unique characteristic of *P. gingivalis* is the expression of peptidyl arginine deiminase (PPAD), capable of citrullinating both host and bacterial peptides (Wegner, Wait et al. 2010). It has been observed that, unlike human PAD2 and PAD4, PPAD can only citrullinate C-terminal arginine and not internal arginine, creating citrullinated peptides that wouldn't occur in the absence of *P. gingivalis*, as human PAD prefers internal arginine substrates. To that end, *P. gingivalis* uses its gingipain enzyme to cleave peptides and generate arginine residues that PPAD would then citrullinate (Abdullah, Farmer et al. 2013). Also, in contrast to human PAD, PPAD is not calcium dependant and this enzyme is capable of auto-citrullination, becoming a citrullinated bacterial protein itself (Quirke, Lugli et al. 2013). These characteristics provide a plausible pathway in which, by presenting neoepitopes to the immune system, *P. gingivalis* (in

susceptible individuals, such HLA-DRB1 carriers) would break the tolerance to citrullinated proteins and lead to the consequent antigen response characteristic in RA patients. (Figure 1. 2).

It is believed that *P. gingivalis* uses these mechanisms for its survival in the gingival pocket. As a result of citrullination, ammonia is generated, which neutralizes acidity and energy is released for *P. gingivalis* to use during anaerobic growth (Nomura 1992) and also as a strategy to suppress the inflammatory activity of C5a by citrullinating it, which reduces its chemottractant nature for neutrophils (Bielecka, Scavenius et al. 2014).



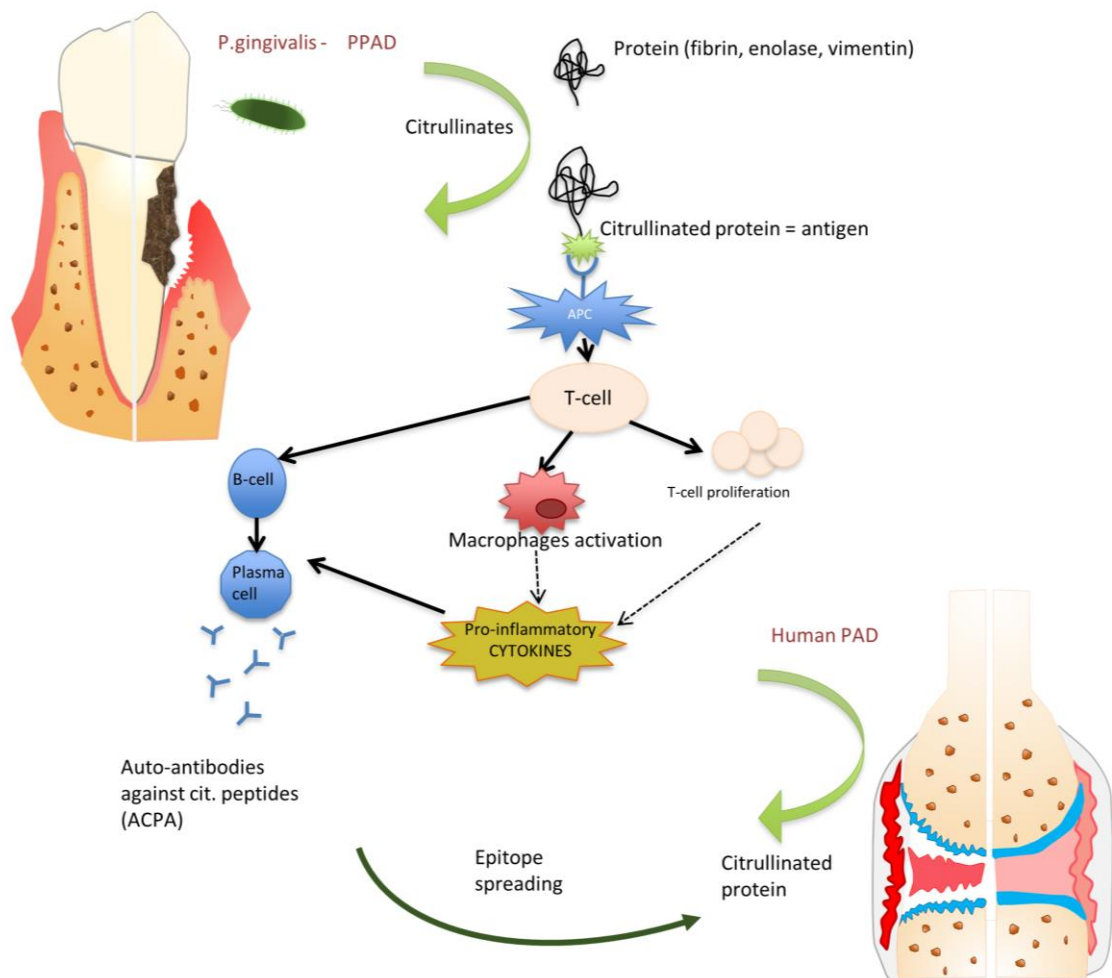


Figure 1. 2 Model of the biological link between rheumatoid arthritis and periodontitis. *Porphyromonas gingivalis* breaking the immune tolerance to citrullinated proteins in rheumatoid arthritis by bacterial citrullination of proteins through PPAD, initiating an immune response leading to loss of tolerance to citrullinated proteins in the joints. Abbreviations: *P. gingivalis*, *Porphyromonas gingivalis*; PPAD, peptidyl arginine deiminase from *P. gingivalis*; APC, antigen presenting cell; TNF, tumor necrosis factor; IL, interleukine; GMCSF, granulocyte-macrophage colony-stimulator growth factor.

Numerous researchers have investigated the role of *P. gingivalis* in RA. The first to suggest that *P. gingivalis* and PPAD could break the tolerance and trigger an antibody response against citrullinated antigens was Rosenstein et al. (Rosenstein, Greenwald et al. 2004). Later, Martinez-Martinez et al. (Martinez-Martinez, Abud-Mendoza et al. 2009) found DNA from *P. gingivalis* in synovial fluid of patients with rheumatoid arthritis, and hypothesized that periodontal pathogens could be the trigger for the autoimmune response in RA.

Wegner et al. showed that *P. gingivalis* citrullinates fibrinogen and alpha-enolase and other peptides from human and bacteria (Wegner, Wait et al. 2010), and Hitchon et al. (Hitchon, Chandad et al. 2010) reported an association between immune responses to *P. gingivalis* and the presence of anti-citrullinated protein antibodies (ACPA) in a population with a high background prevalence of RA predisposing HLA-DRB1 alleles. They suggested that this gene-environment interaction might be the cause of the break of tolerance of the immune system to citrullinated proteins that leads to RA. A study by Mikuls et al., has shown that high levels of anti-*P. gingivalis* antibodies in RA subjects correlates with levels of anti-CCP IgM and IgG suggesting that this bacterium plays a role in risk and severity of RA (Mikuls, Thiele et al. 2012).

Interestingly, it has been observed that the periodontium of patients with no signs of RA but diagnosed with periodontitis express citrullinated proteins (Nesse, Westra et al. 2012) and serum of periodontitis patients contains higher levels of antibodies to citrullinated and non-citrullinated human peptides compared to

controls (de Pablo, Dietrich et al. 2014). De Pablo and collaborators suggest that the greater citrullination and loss of tolerance to citrullinated and uncitrullinated peptides that occur in periodontitis, may lead to epitope spreading to citrullinated epitopes, and evolve to Rheumatoid Arthritis.

Based on these findings, researchers propose that bacterial and human PADs could be a powerful target for therapy in RA (Mangat, Wegner et al. 2010). Moreover, it has been hypothesized that by removing subgingival plaque with periodontal therapy, the load of *P. gingivalis* and PPAD can be decreased, and therefore create a reduction of the generation of antigens and weaken the autoimmune response in RA.

#### 1.5.2. Other bacterial citrullination

More recently, other periodontal bacteria have been investigated as a source of citrullination and possible link between periodontitis and RA. Among the different periodontal pathogens and oral commensals investigated in a recent study, only *Aggregatibacter actinomycetemcomitans* could induce hypercitrullination of human neutrophils (Konig, Abusleme et al. 2016). *A. actinomycetemcomitans* induces neutrophil hypercitrullination through the secretion of leukotoxin A (LtxA), a bacterial pore-forming toxin that induces calcium influx and subsequent hyperactivation of PAD enzymes in the neutrophil. Interestingly, the effect of human lymphocyte antigen–DRB1 shared epitope alleles on auto-antibody positivity was limited to RA patients who were exposed to *A. actinomycetemcomitans*. These studies identify the periodontal pathogen *A. actinomycetemcomitans* as a candidate bacterial trigger of autoimmunity in RA



### 1.5.3. Citrullinated human proteins targeted in RA

Anti-cyclic citrullinated peptide (CCP) kits are regularly used by rheumatologists to measure antibodies against citrullinated proteins and diagnose patients as ACPA+ and ACPA- which can anticipate disease severity and treatment response (Seegobin, Ma et al. 2014). This test uses a synthetic citrullinated cyclic peptide as an antigen to capture antibodies against any citrullinated proteins present in patient sera. Investigating the proteins that are citrullinated in RA is important, as it can help understand the development of the autoimmune response and development of the disease. To date, numerous studies have reported that proteins such as Fibrinogen, Vimentin, Enolase and Tenascin are targeted in RA and antibodies against these citrullinated proteins are associated with severity of the disease (Montgomery, Venables et al. 2013), (Nielen, van Schaardenburg et al. 2004) (Raza, Schwenger et al. 2016).

Fibrinogen is an acute-phase reactant protein, synthesized in the liver, involved in clot formation by its conversion to fibrin by thrombin. Fibrinogen is elevated in numerous inflammatory diseases, such as periodontitis and RA, and antibodies against citrullinated fibrinogen are elevated and correlated to clinical and laboratory RA parameters (Zhao, Okeke et al. 2008) (Gilliam, Reed et al. 2011).

Vimentin is a type III intermediate filament, responsible for maintaining mesenchymal cell (mucoid connective tissue) structure. In RA, antibodies against citrullinated vimentin and mutated vimentin (MCV) are used as diagnostic tool,

especially in RF negative patients (Dejaco, Klotz et al. 2006). These antibodies are found before the onset of RA and also estimate bone destruction (Mathsson, Mullazehi et al. 2008). In systemically healthy periodontitis patients, antibodies to cit-Vimentin are elevated compared to controls (de Pablo, Dietrich et al. 2014).

Enolase 1, commonly known as  $\alpha$ -enolase, is a glycolytic enzyme present in most tissues that has numerous functions (surface protein, tumor supresor, oxidative, stress, etc). Antibodies against enolase are elevated in numerous autoimmune diseases such as RA, ulcerative colitis and Crohn's disease (Pancholi 2001). In the joints of patients with RA, enolase is up regulated in response to inflammation. While high levels of anti-enolase are associated with a variety of autoimmune diseases, antibodies against citrullinated enolase are only found in RA (67% of RA, only 3% of healthy individuals) (Lundberg, Kinloch et al. 2008) and correlated with disease severity (Kinloch, Tatzer et al. 2005). Elevated antibodies against cit-enolase 1 are higher in CCP positive and smoking RA patients (Mahdi, Fisher et al. 2009) and are also present in early-RA patients (Saulot, Vittecoq et al. 2002). Interestingly, the human enolase and *P. gingivalis* enolase have been found to share 82% homology, with antibodies to CEP-1 cross-reacting to *P. gingivalis*-enolase (Lundberg, Kinloch et al. 2008). Therefore, it has been hypothesized that this similarity and crossreactivity could be the link between *P. gingivalis* and RA (Lundberg, Wegner et al. 2010).

Tenascin-C is a large extracellular glycoprotein that stimulates inflammation activating toll-like receptor 4 (TLR4). This protein is absent in most healthy tissues

and up-regulated during inflammatory stages, such as in the RA joint, maintaining tissue damage and inflammation (Midwood, Sacre et al. 2009). Antibodies against an epitope of citrullinated TNC5 (cTNC5) have been detected in 40-50% of RA patients, correlated with bone erosion and associated with RA development in early stage of the disease (Raza, Schwenzer et al. 2016, Schwenzer, Jiang et al. 2016).

#### 1.5.4. *P. gingivalis* antigens targeted in RA:

It has been proposed that proteins from *P. gingivalis* could be recognized as an antigen by the host and initiate an immune response that, in RA, could break the tolerance to human antigens such as citrullinated proteins. For this reason, recently, researchers have investigated the antibodies against some *P. gingivalis* -antigens and its role in RA.

Antibodies against citrullinated PPAD (specifically epitopes CPP3 and CPP5) are increased in ACPA+ RA patients as well as in periodontitis patients (CPP5) (Quirke, Lugli et al. 2013). In RA and pre-RA patients, anti-CPP3 antibodies are elevated (Johansson, Sherina et al. 2016), although other authors did not find an association between anti-PPAD and early RA (Fisher, Cartwright et al. 2015).

Gingipain enzymes are a family of trypsin-like cysteine proteinases that are secreted by *P. gingivalis* and contribute to its virulence, as well as to the pathogenesis of periodontitis and is the most potent virulent factor produced by *P. gingivalis* (Potempa, Sroka et al. 2003). Antibodies against gingipain are

significantly increased in periodontitis but are not clearly correlated with RA (Quirke, Lugli et al. 2014) (Kharlamova, Jiang et al. 2016).

*P. gingivalis* enolase, due to its similarity with human enolase, has been proposed to be the link between RA and PD in which, antibodies against human cit-enolase cross-react with a conserved sequence on citrullinated *P. gingivalis* enolase. Lundberg and colleagues hypothesized that this molecular mimicry between human and bacterial enolase is involved in the etiology of RA (Lundberg, Wegner et al. 2010).

Considering these results, researchers support the hypothesis that *P. gingivalis* -antigens might break tolerance in RA, and if proven, these epitopes could be targeted in RA patients (Quirke, Lugli et al. 2014).



#### 1.5.5. The role of the oral microbiome in RA

The human microbiome is defined as the genes carried by the particular community of microorganisms that live in and on the human body. This human microbiota is diverse and lives in symbiosis with the human body and the balance of this ecosystem impedes the appearance of pathogens, as they compete for the resources available in the environment. This "normal" microbial community has been investigated with cultivation techniques, which is not applicable for most of the complex ecosystems in our body, such as the oral cavity. However, the Human microbiome project using DNA-based technologies has opened a new horizon in the understanding of the microbes that live in and on us (Turnbaugh, Ley et al. 2007).

Bacterial identification through the study of the DNA can target the entire DNA present in a sample (genome) or can target a specific gene. By studying highly conserved genes, such as the gene that codes for the 16 S ribosomal RNA (16S rRNA), we can identify the different species according to the different sequences of this gene. The gene 16S rRNA, which codes for the small part of the ribosome complex, is the most analyzed as is contained in the genome of all bacteria and is never translated into a protein, as it is structural RNA. This gene has conserved regions, which are very important and carried by all bacterial species, and other regions with mutations according to the different species, the variable regions. Sections of the genes that contain both variable and conserved regions allow researchers to differentiate between species (Morgan and Huttenhower 2012).

To investigate a particular region, primers (a manufactured short nucleotide sequence of 12 nucleotides used as a probe) are added to match the conserved region just before and after the selected section that need to be sequenced. To study the oral microbiome, Kumar and collaborators concluded that use of 4 primers on the 2 regions V1 (27F)-V3 (534R) and V7-V9, are the best to identify the largest amount of species (Kumar, Brooker et al. 2011). While the Sanger method used to be the gold standard, it is very slow and expensive, and Next Generation Sequencing (NGS) technologies use regions to identify taxa, reducing the cost and increasing the speed (Goodwin, McPherson et al. 2016). Different high-throughput sequencing platforms have been established since the mid-2000s although each method comes with limitations, that lead to the development of pipelines to reduce errors and biases produced as a results of these methods (Dabdoub, Fellows et al. 2016) (Caporaso, Kuczynski et al. 2010) (Edgar 2013).

Using these technologies, researchers have found that individuals with non-infectious systemic diseases (especially autoimmune diseases such as Cronh's disease, Colon cancer and food allergies) have a different human microbiome. The study of this disease-associated species is fundamental to elucidate the role of oral microbiome and its potential use in diagnosis (Blaser<sup>1</sup> 2012). While the cause of RA remains unknown, it has been hypothesized that oral microbiota might have a role in its etiology and pathogenesis (Wegner, Wait et al. 2010) (Brusca, Abramson et al. 2014) (Scher, Bretz et al. 2014),.

The oral cavity is the site of the body with the richest core microbiome, which is defined as the common microbiome shared by most or all subjects sampled (Huse, Ye et al. 2012). Although the decreases in cost and increase in speed of Next-Generation Sequencing (NGS) technologies have resulted in an explosion of 16S data, the current literature available on the oral microbiome is more limited compared to the gut microbiome (Turnbaugh, Ley et al. 2007).

As the data acquired from microbiome studies is so complex, novel and specific analysis is required. Alpha diversity is used to measure the diversity of bacteria within a group or sample, which can be determined by the richness (how many different species there are) and the evenness (the differences in abundance of those species). To measure differences between bacteria diversity between groups, Beta diversity is used to study the differences between habitats (Morgan and Huttenhower 2012).

Currently, two next generation sequencing studies have investigated the oral microbiome in RA (Zhang, Zhang et al. 2015) (Scher, Ubeda et al. 2012). In the metagenome study of Zhang and collaborators, they found that subgingival, oral and gut microbiome are altered and certain species were identified as potential targets or diagnostic tools for RA. In the study from Scher and colleagues, in early RA patients some bacterial taxa were identified to be significantly different in abundance but overall measures of diversity were not different to systemically healthy controls.

However, in these studies, more than 75% of the new-onset RA and RA patients studied had moderate to severe periodontitis (Scher, Ubeda et al. 2012) or periodontal status was not considered (Zhang, Zhang et al. 2015). Since the oral microbiome is known to be altered in periodontitis, the contradicting results from these studies might be confounded by the presence of periodontitis. Thus, it is necessary to examine the oral microbiome in periodontally healthy RA patients and study the possible differences compared to systemically healthy controls to verify if this described shift in the oral microbiome is due to RA and not just as a consequence of the high prevalence of periodontitis in this group.

It has also been proposed that, in RA patients, the use of DMARDs and biologic therapies restores the dysbiotic oral microbiome (Zhang, Zhang et al. 2015). However, the effect of periodontal therapy on the oral microbiome has not been fully investigated using NGS techniques and the effect in RA patients is not known.

#### 1.5.6. The cytokine imbalance in RA and Periodontitis

In RA, there is an imbalance in the main cytokine system (Feldmann, Brennan et al. 1996) in a similar manner that occurs in periodontitis patients (Gemmell, Carter et al. 2001). Understanding the cytokines involved in the pathomechanisms of RA has led to a revolution in the therapeutics of this disease in the last decade (Azizi, Jadidi-Niaragh et al. 2013) and therefore, this cytokine imbalance could be the biological link between RA and periodontitis.

The human microbiota lives in cross-talk with the human immune system and can trigger an immune response to their benefit. In periodontitis, cytokines such as IL-1 $\beta$  can help the growth of some species such as *A. actynomicetemcomitans*, which in the presence of this cytokine, decrease their metabolism and increase their biomass (Pöllänen, Paino et al. 2013).

Investigating the cytokine profile and correlating this with the microbial communities would help understand the complex mechanisms that link periodontitis, and subgingival microbiota with the aetio-pathogenesis of RA.

The imbalance between pro-inflammatory and anti-inflammatory cytokines and growth factors is comparable: In RA and periodontitis high levels of IL-6 and TNF- $\alpha$  (now named TNF) systemically and locally are found, which are known to activate the inflammatory response. On the other side, lower levels of IL-10 and growth factor-b, are known to have anti-inflammatory effect are found to be decreased in RA and PD (Kobayashi and Yoshie 2015).

Of special importance is the pro-inflammatory cytokine TNF, as it plays a key role in the immune response for both RA and periodontitis. TNF modulates the inflammatory response as it can increase the inflammatory response by binding to the receptor p55 (TNF receptor type 1; CD120a), whereas binding to receptor p75 (TNF receptor type 2; CD120b) attenuates the inflammatory response (Peschon, Torrance et al. 1998). In periodontitis, TNF has been associated with the breakdown of connective tissue attachment and bone loss (Assuma, Oates

et al. 1998). RA patients suffering with periodontitis have higher levels of TNF compared to non-periodontitis and these levels are positively correlated with RA severity (Kobayashi and Yoshie 2015).

To date, several small and short studies have evaluated the effect of TNF inhibitors in periodontitis and RA (Ortiz, Bissada et al. 2009) (Pers, Saraux et al. 2008) (Mayer, Balbir-Gurman et al. 2009) (Üstün, Erciyas et al. 2013) (Kobayashi, Yokoyama et al. 2014). Although the authors concluded that anti-TNF therapy could benefit periodontal status, they suggest that it is very difficult to predict the harm that may result from targeting cytokines and the study from Pers and colleagues, anti-TNF therapy increased gingival inflammation in RA patients, although decreased clinical attachment loss. Therefore, it is not clear if anti-TNF therapy is beneficial for periodontitis.

Interestingly, periodontal therapy has been shown to reduce serum TNF levels (O'connell, Taba Jr et al. 2008) (Nishimura, Iwamoto et al. 2003). For this reason, some researchers highlight the importance of investigating periodontal therapy as a possible safe and easy way to reduce inflammation in systemic inflammatory conditions such as RA.

Likewise, other cytokines such as IL-6 are associated with both RA and periodontitis. IL-6 is a pro-inflammatory cytokine with numerous functions and anti-IL-6 therapy is shown to reduce periodontal parameters such as BoP, GI and CAL (Kobayashi, Okada et al. 2014). A variety of studies have shown that

periodontal treatment reduces serum levels of cytokines like IL-6 (Shimada, Komatsu et al. 2010) (D'aiuto, Nibali et al. 2005) (Duarte, da Rocha et al. 2010). In RA patients suffering with periodontitis, IL-6, MMP-8 and PGE<sub>2</sub> were reduced in the gingival crevicular fluid after periodontal therapy (Kurgan, Fentoğlu et al. 2016). These findings suggest that periodontal therapy could be a non-pharmacological method to reduce the pro-inflammatory cytokines locally and systemically in patients with RA.

#### 1.5.7. Other theories on the link between RA and periodontitis

##### 1.5.7.1. Neutrophils and neutrophil extracellular traps

Neutrophils are one of the first white blood cells to arrive at the site of infection. They represent 60% of all leukocyte cells and their main functions include phagocytosis of microorganisms, recognition and activation of humoral immunity. In healthy periodontium, the gingival crevicular fluid (GCF) contains neutrophils, which contribute towards maintaining a balanced gingival microbiota. If an invasion from a pathogen is detected, macrophages in the gingival tissue activate receptors in the vascular endothelium to facilitate neutrophil recruitment and diapedesis to the site of infection. Once in the site, neutrophils phagocytose the microbe to destroy it with their lethal enzymes contained in the granules. This process happens within the safe confines of the membrane of the neutrophil cell. Then the neutrophil enters into apoptosis and releases signals that attract macrophages to remove the apoptotic bodies. However, when activated by

specific signals, neutrophils release their enzymatic content to the exterior of the cell, together with its DNA, forming a net with the aim of trapping and rapidly killing the microbes, called neutrophil extracellular traps (NETs). Although this is an effective tool to kill bacteria (Brinkmann, Reichard et al. 2004), the release of this contents, along with Reactive Oxygen Species (ROS) provokes collateral damage to the surrounding tissues and exacerbates the inflammatory response and expose possible autoantigens (Liou and Campbell 1996).

Aberrant NETs release has been observed in RA synovial fluid and serum compared to healthy controls and Osteoarthritis, and NET release was correlated with ACPA and RF levels (Khandpur, Carmona-Rivera et al. 2013). Interestingly, in RA patients, NETs were induced by IL-17, TNF and ACPAs such as anti citrullinated-Vimentin antibodies, contained proteins and neutrophils undergoing NETosis, in turn, externalized citrullinated antigens and activated the release of other pro-inflammatory cytokines (Khandpur, Carmona-Rivera et al. 2013).

Moreover, it has been shown that PAD-4 activation is necessary for NET production. In PAD-4 deficient mice, neutrophils did not undergo NET formation, compared to the control group. Also in this study, it was noticed that histone citrullination by PAD4 leads to chromatin decondensation, facilitating the production of NETs (Li, Li et al. 2010). These results suggest that in RA, NETs contribute to the maintenance of the inflammatory response and become a source of citrullinated antigens in RA.



Elevated NET formation also occurs in periodontitis patients. This might be due to chronic exposure to periodontal pathogens that results in an elevated number of apoptotic neutrophils which, overtime, would lead to increase inefficient chemotaxis and elevated NET production (Cooper, Palmer et al. 2013).

Taken together, these results suggest that, in periodontitis, a chronic exposure of gingival tissues to PAD4 and citrullination, may lead to breakdown of tolerance against citrullinated peptides in susceptible individual, leading or contributing to RA pathogenesis. NET generation not only contributes to periodontal and RA pathogenesis, but also provides a potential causal link between these two conditions (Figure 1. 3).

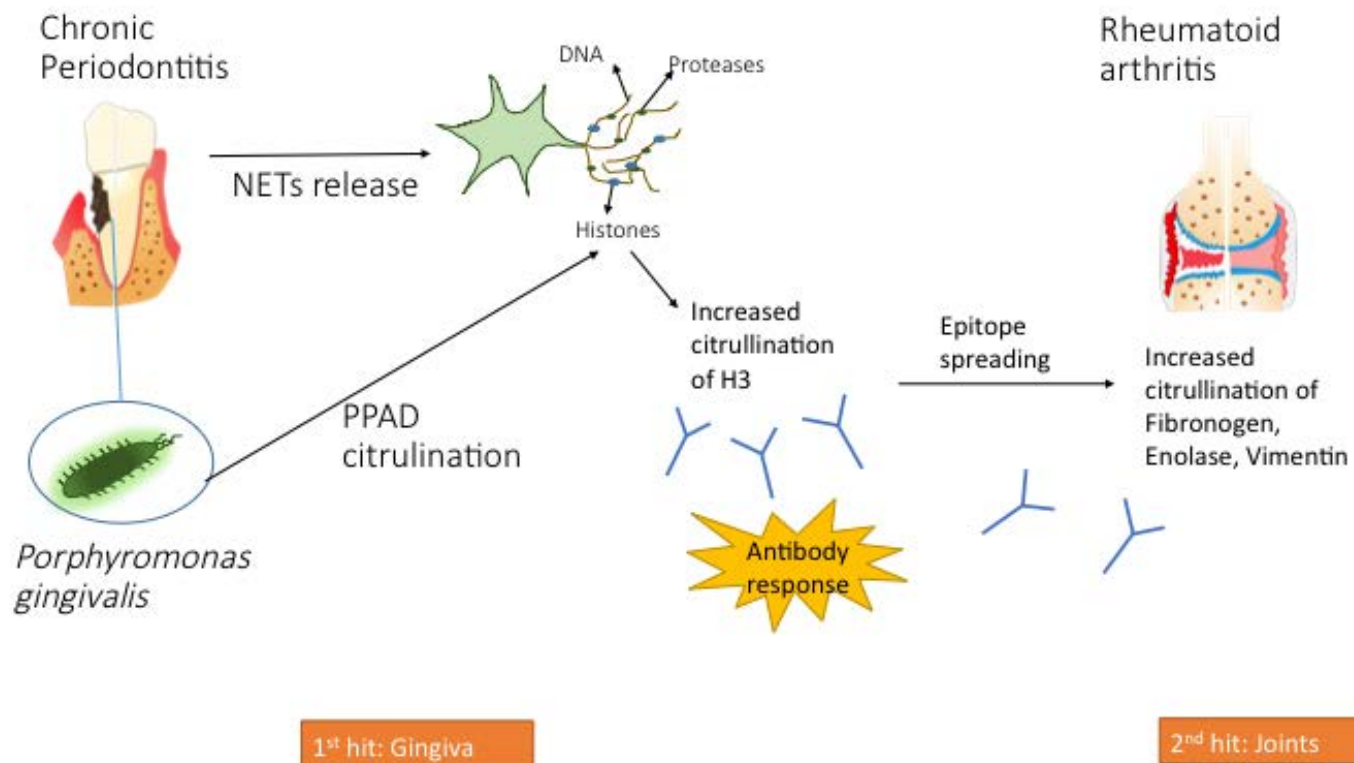


Figure 1. 3 Hypothesis of the role of NETs in the connection between RA and periodontitis. Abbreviations: NETs, neutrophil extracellular traps; PPAD, peptidyl-arginine deiminase from *Porphyromonas gingivalis*.

#### 1.5.8. "Two-hit" model

Golub et al. were the first researchers to describe a theory in which the association between periodontitis and systemic diseases could be explained by a "two hit" model. In this theory, a first "hit" occurs within the periodontum, initiated by an infection or inflammation of gingiva, that activates a destructive inflammatory cascade in the periodontal tissues. In susceptible patients, a second systemic "hit" occurs, characterized by increased serum levels of inflammatory cytokines that amplify the inflammatory cascade, lead to the release of local and systemic inflammatory mediators (cytokines and prostaglandins), a rise of RANKL and MMPs, along with other destructive proteinases, that leads to bone destruction and periodontal breakdown (Golub, Payne et al. 2006).

Years later, a similar "two hit model" was described to explain the breakdown of tolerance to citrullinated proteins in RA patients triggered by smoking. Based on this model, in the first hit, antibodies to citrullinated proteins are produced due to the break of tolerance to these proteins, which are citrullinated due to cigarette smoking. In a second hit years later, ACPAs are released systemically and specifically in the joint structure, as it has been observed that synovium is especially sensitive to inflammatory stimuli (Farquharson, Butcher et al. 2012).

Similarly to smoking, periodontitis could represent a first hit of citrullinated peptides. In people who are genetically susceptible, a first hit of chronic periodontitis could break the tolerance to citrullinated proteins, due to abnormal

and bacterial citrullination by *P. gingivalis*. These bacterial citrullinated proteins are recognized as an antigen and presented to B cells, which secrete antibodies against proteins citrullinated by *P. gingivalis*. The transition to the autoimmune response occurs years before the clinical onset of RA, due to the recognition of new epitopes through epitope spreading, which perpetuates and amplifies autoimmune diseases. Activated B cells can process and internalize molecules closely associated with the initial antigen (such as CEP and *P. gingivalis* enolase), via endocytosis and present the new peptides to T cells. This way, B cells can act as antigen presenting cells for peptides that are different from the antigen that initiated the immune response (Murphy 2012). Through this epitope spreading, a second auto-immune hit could happen years later in the joint structure, where ACPAs are released against a variety of human citrullinated peptides such as fibrinogen, enolase and vimentin. This model describes a primary "hit" of chronic inflammation via chronic periodontitis followed by a secondary "hit" in the joint, that induces rheumatoid and an increased inflammatory response (Kaur, White et al. 2013) (Lundberg, Wegner et al. 2010) (Figure 1. 4).

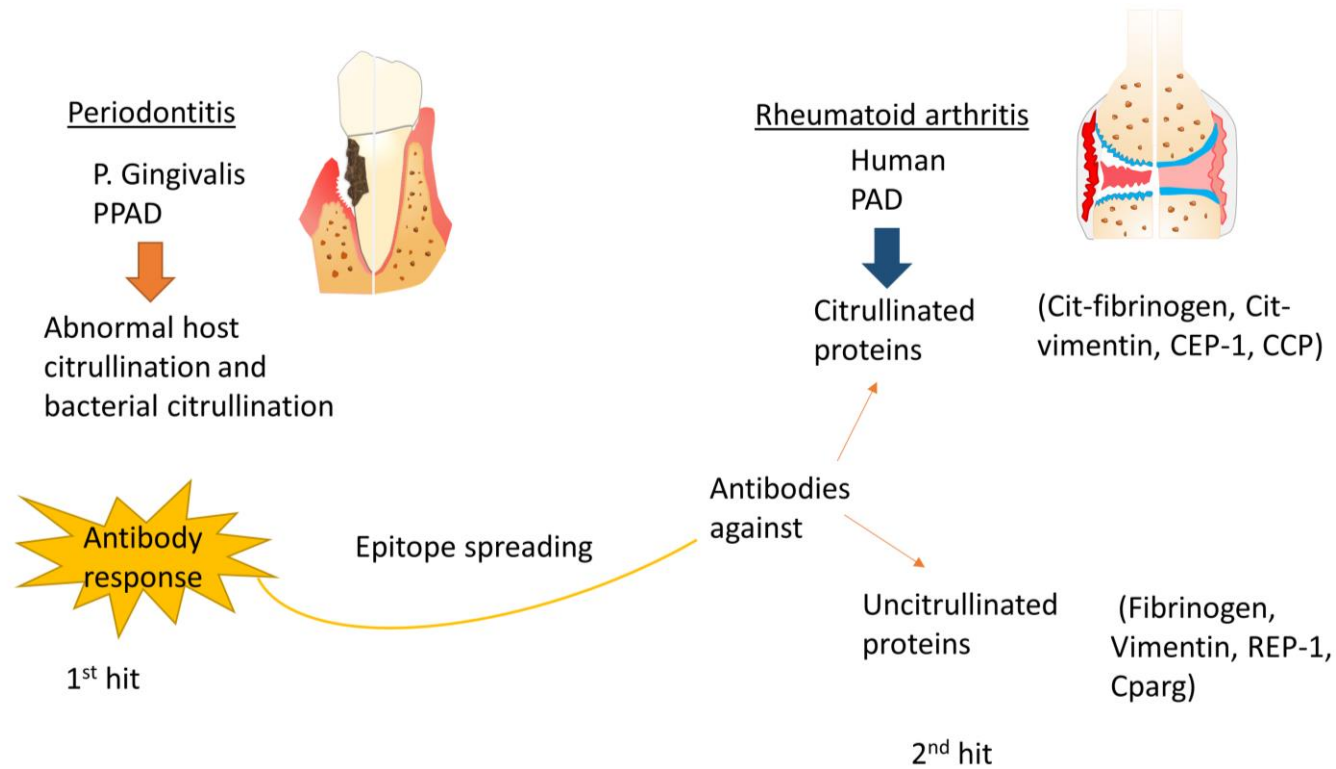


Figure 1. 4 Two hit model proposed to explain the link between periodontitis and RA. Abbreviations: PAD, peptidyl arginine deiminase ; PPAD, PAD from *P. gingivalis*; Cit- citrullinated; CEP, cit-enolase ; REP, arginated (non-cit) enolase; CCP, cyclic citrullinated peptide.,

Although promising, none of the theories described explain why this inflammatory response that is hypothetically initiated or exacerbated by periodontitis would be specific to the joint structure in RA. While it seems that there is an epidemiological relation between the two conditions, it is not proven whether this is coincidental or due to common risk factors or if there is indeed a causal relationship. Future research is needed to clarify the gaps in the literature in this matter since, if proven, periodontitis prevention and therapy could ameliorate the progression of RA.

#### 1.6. Thesis aims

The role of periodontitis in RA severity and pathogenesis has not yet been revealed. The results from the small clinical trials published, although promising, do not show enough evidence to conclude that periodontal therapy could help improve RA parameters. Using a variety of biological samples collected from a pilot clinical trial (OPERA, further explained in methods section Chapter 2) the overall aim of this thesis is to investigate the role of periodontitis in RA and the effect of periodontal therapy not only in clinical parameters, but on different markers that could be related to RA severity or elucidate the role of periodontitis in RA.

Objective 1: To investigate the antibody response to citrullinated and un-citrullinated peptides in sera of patients with RA and Periodontitis compared to RA alone, Periodontitis alone and the systemically and periodontally healthy controls and possible changes after periodontal treatment.

Objective 2: To investigate the antibody response to periodontal bacteria antigens in sera of patients with RA and Periodontitis compared to RA alone, Periodontitis alone and the systemically and periodontally healthy controls and possible changes after periodontal treatment.

Objective 3: To compare the subgingival microbiota of patients with RA and periodontitis, RA alone, periodontitis alone and the systemically and periodontally healthy controls and effect of periodontal therapy.

Objective 4: To investigate the differences in the cytokine response in gingival crevicular fluid in patients with periodontitis and RA (compared to controls: RA alone, periodontitis alone) and how non-surgical periodontal therapy changes immune response signatures.

## **2. MATERIALS AND METHODS**



## 2.1. Study volunteers

For the purpose of this thesis, biological samples collected from two different pilot clinical studies were analyzed. The three types of human samples studied were serum, gingival crevicular fluid (GCF) and plaque. Different sample sizes were selected from these cohorts according to resources and availability of the samples. Figure 2.1 shows the study populations and the quantity of samples utilized for the different analyses.

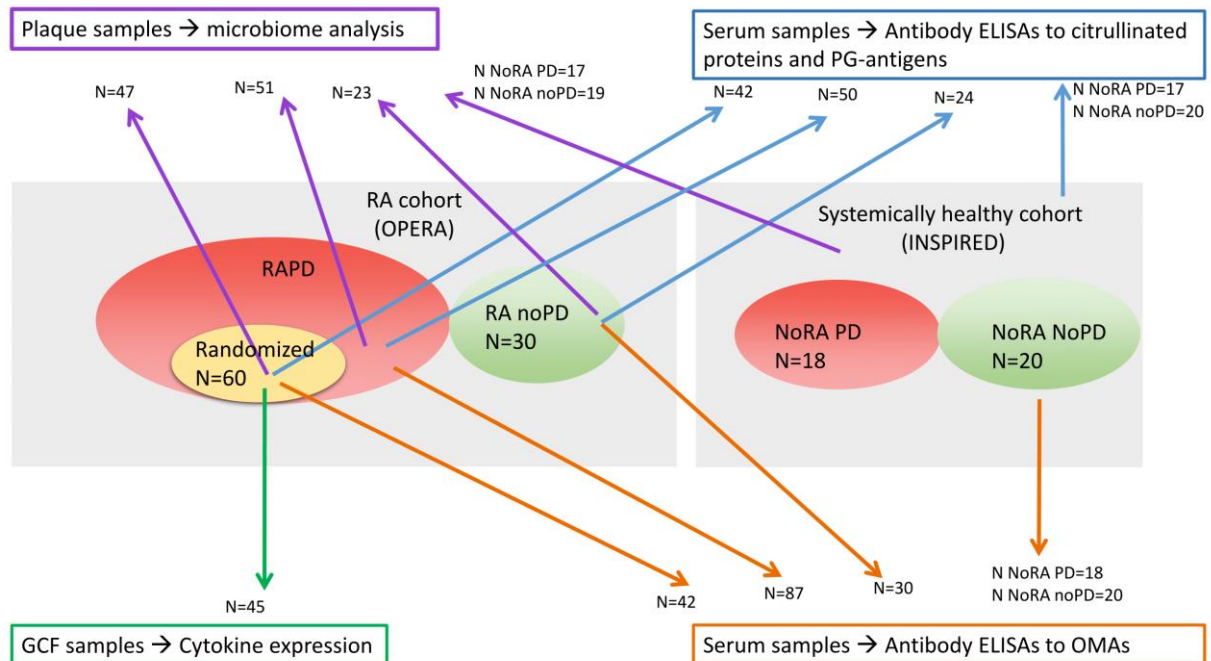


Figure 2. 1 Study population included for the different studies of this thesis. Plaque, and serum samples were collected (methods described in section 2.1) for the analysis of the immunological and microbiological relationship between RA and periodontitis. When samples were available, the same patients were investigated from each baseline group, which overlapped in the majority of the experiments. Patients diagnosed with mild periodontitis are not represented in this diagram and are excluded for baseline comparisons. Abbreviations: N, number of patients; RA, rheumatoid arthritis; PD, periodontitis. ELISA, enzyme-linked immunosorbent assay; OMA, outer-membrane antigen; PG, *Porphyromonas gingivalis*.

### 2.1.1. Rheumatoid arthritis patient volunteers: OPERA clinical trial

To address the objectives of this thesis, samples were collected as part of the OPERA study (Outcomes of Periodontal Therapy in Rheumatoid Arthritis). Approval for this study was obtained from the South Birmingham Research Ethics Committee, UK (Approval codes: OPERA - 11/WM/0235) and the study was funded by the National Institute of Health Research (NIHR) (Grant Reference Number PB-PG-0609-19100).

The described OPERA study was a randomized feasibility clinical trial which aimed to collect feasibility information for a future pivotal study to address if periodontal therapy could improve RA clinical parameters. Although the clinical data of this study could not answer the final question, a range of periodontal and RA clinical samples were collected from a large number of RA patients with different periodontal characteristics and from the longitudinal part of the trial. These samples were investigated in this thesis to examine the involvement of the immunological and microbiological characteristics in the interplay between RA and PD.

#### 2.1.1.1. Recruitment of OPERA study

Recruitment started in January 2014 and finished in October 2015. Patients diagnosed with Rheumatoid Arthritis (according to the revised 1987 ACR criteria) from three different hospitals in Birmingham (Queen Elisabeth Hospital, Heartlands Hospital and City and Sandwell Hospital) were contacted and informed about the study by post or by nurses in the rheumatology department.

If patients were interested in the study, full verbal explanation and written information was given in a Patient Information Sheet. If the patients were willing to take part on the study, they signed an Informed Consent Form and were given a date to attend an appointment at the Birmingham Dental Hospital for a screening visit.

A total of 296 patients from the 3 different hospitals were consented to the study. However, 98 patients (33%) either did not attend this appointment or dropped out at the recruitment stage (Figure 2. 2 )

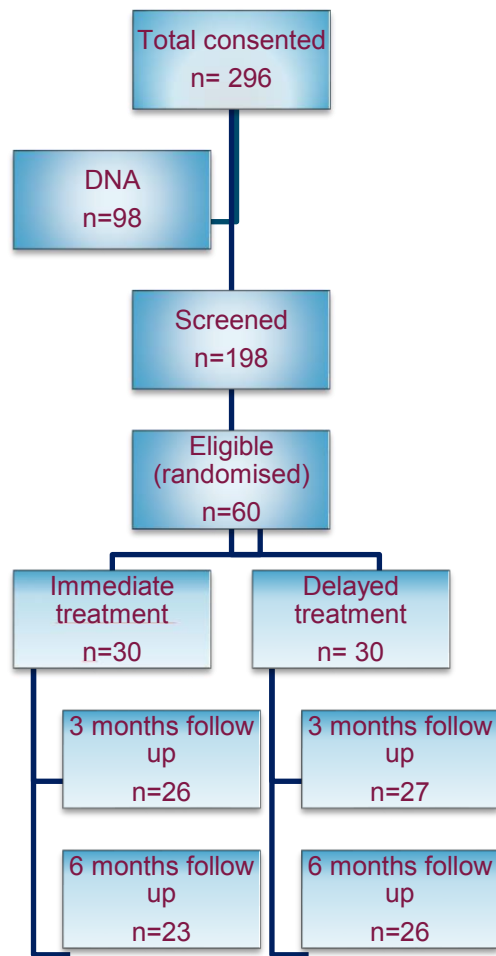


Figure 2. 2 CONSORT diagram of the OPERA clinical trial. Abbreviations: OPERA, Outcomes of periodontal therapy in rheumatoid arthritis; n, number of patients per group; DNA, did not attend the appointment.

#### 2.1.1.2. Screening appointment: clinical assessment

A total of 198 patients were screened for the study. The screening appointment started with an assessment of the general health using a detailed medical questionnaire and taking the blood pressure, weight and height of the patient.

The assessment was followed by an examination of the swelling and pain (tenderness) of 28 joints according to the disease activity score (DAS) 28 (Prevoo, Van't Hof et al. 1995): the shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and the knees. To calculate the DAS 28 a visual scale analogue (VAS) was given to the patients and used to assess the negative impact that RA had in their life for the last 2 weeks, in a scale from 0 to 100. These parameters together with the erythrocyte sedimentation rate (ESR) value from the hospital notes, was entered in the formula for the calculation of the DAS28:

$$\text{DAS28} = 0.56 * \text{sqrt}(\text{tender28}) + 0.28 * \text{sqrt}(\text{swollen28}) + 0.70 * \ln(\text{ESR}) + 0.014 * \text{VAS}$$

For the assessment of periodontal status, a Periodontal Pocket Chart was performed recording the clinical attachment level, bleeding on probing, pocket depth and gingival recession. To guarantee consistency, the same operator which was calibrated and trained by expert periodontists from the periodontology

department (the author of this thesis) performed the periodontal examination at baseline and in the review visits of the longitudinal part of the trial.

Established questionnaires were used to assess the patient physical function, to assess quality of life in relation with oral health, and the RA and general health status: Arthritis impact measurement scales 2 (AIMS2), Multidimensional Health Assessment Questionnaire (R808-NP2), Multidimensional assessment of fatigue scale (MAF-scale), Patient Health Questionnaire (PHQ-9), Oral health impact profile (OHIP) and Euro Quol questionnaires (Slade 1997).

Covariate data was collected at baseline such as age, gender, ethnicity, socio-economic status, education, marital status, smoking habits, alcohol consumption, comorbidities and medication.

#### 2.1.1.3. Sample collection

During the screening appointment, if the patient accepted and consented, samples of blood, saliva, gingival crevicular fluid (GCF) and subgingival plaque were collected. If the patients participated in the longitudinal study, these samples were collected also after 3 and 6 months of the hygienist visit.

##### 2.1.1.3.1. Gingival crevicular fluid

Gingival crevicular fluid (GCF) was collected after carefully drying the area with low-pressure air and cotton roll isolation to avoid saliva contamination. The

collection was performed inserting PerioPaper® strips in the periodontal pocket for 30 seconds from 4 selected sites in the upper jaw: mesial of the first premolar and mesial of the first molar of each side. If selected teeth were not present or there was contamination, adjacent teeth were selected and annotated to ensure consistency if GCF was collected in future visits. After measuring the volume of GCF in a Periotron® 8010 (Oraflow Inc., USA), PerioPapers were immediately snap-frozen with liquid nitrogen and stored at the end of the screening visit at -80°C in a 1.5ml Star Lab tube (E1480-0306).

#### 2.1.1.3.2. Saliva samples

Samples of saliva were collected using a sterile marble to stimulate saliva production for 5 minutes. The volume of saliva produced during the initial 5 minutes was used for the calculation of the salivary flow rate (ml/min). The saliva collected in a Falcon tube (Corning Incorporated, USA) was immediately stored in wet ice and taken to the laboratory once the appointment finished where samples were centrifuged at 1000 rcf at 4°C for 10 minutes (Rotina 380R, Hettich®), supernatant and pellet separately were transferred into a 1.5ml cryogenic vial (Star Lab, UK) and stored at -80°C for future analysis.

#### 2.1.1.3.3. Blood samples

Blood samples (approximately 30 ml) were collected from the antecubital fossa into heparinized tubes to separate the plasma (E1390-0304, Star Lab) and 4



serum-separating tubes (E1480-0302, Star Lab) vacutainer® 5ml tubes. Plasma tubes were immediately stored on ice while serum tubes were left undisturbed for 15 to 30 minutes before storing on ice. After the appointment, samples were taken to the laboratory and tubes were centrifuged at 1000 rcf at 4°C for 30 minutes (Rotina 380R, Hettich®), aliquoted into 1.5 ml cryotubes and stored at -80°C.

#### 2.1.1.3.4. Plaque samples

Subgingival plaque was collected after careful removal of supragingival plaque using a cotton pellet. Subgingival plaque collection was performed using a Gracey curette from 6 representative sites, one per sextant as distant as possible. These samples were collected from 3 deep sites (>3 mm) and from 3 shallow sites (<3mm). If patients had healthy periodontium or no deep sites, 3 shallow sites were selected. The total plaque collected per patients was pooled into 1 ml Eppendorf tubes containing 1ml of Tris-EDTA (50 mM Tris, Ethylenediaminetetracetic acid, pH 7.4). These sites were annotated to ensure consistency if plaque was collected in future visits. Subgingival plaque from deep sites (>3 mm) and shallow sites (<3 mm) were collected separately for each patient in the systemically healthy group with periodontitis, to allow the comparison between the two locations.

#### 2.1.1.4. Inclusion and exclusion criteria

The main purpose of these criteria was to assure a sufficient disease activity for both RA and periodontitis (Table 2.1 and Tabel 2.2). To that end, a compilation of validated questionnaires were given to patients, disease activity score (DAS28) was calculated, and full dental and periodontal assessment was performed during the screening visit. The main inclusion criteria had the aim to assure enough RA and periodontitis activity, which standardized through a DAS 28 higher than 3.2, and moderate to severe periodontal disease. To include patients with a sufficient level of inflammation, cumulative probing depth higher than 40 mm was defined as inclusion criteria, this being the sum of the highest probing depth per tooth when probing depths were higher or equal to 4 mm. The purpose of the exclusion criteria was to assure all patients started the study with stable medication (treatment with DMARD for >3 months and stable dose for >2 months). The exclusion criteria aimed to reject patients with other types of periodontal disease and rheumatic or systemic autoimmune disease other than RA. Also patients that had underwent periodontal treatment (within 12 prior to baseline), surgical procedures (within 12 weeks prior to baseline) or glucocorticoids injections (within 4 weeks prior to baseline) were excluded from the study.

Table 2. 1 **Inclusion and exclusion criteria for the OPERA** (Outcomes of Periodontal Therapy in Rheumatoid Arthritis) study. Abbreviations: RA, Rheumatoid Arthritis; ACR, American College of Rheumatology; DAS, Disease Activity Score; DMARD, Disease Modifying Anti-Rheumatic Drug; CAL, Clinical Attachment Loss.

Inclusion	Exclusion
Age 18+, accepting to participate in the study and willing to sign a consent form	Other rheumatic autoimmune diseases different from RA, or significant systemic involvement secondary to RA
Diagnosed according to the revised 1987 ACR criteria for the classification of rheumatoid arthritis (RA)	Other inflammatory joint disease (past or current) different from RA
DAS28 $\geq 3.2$	Juvenile idiopathic arthritis (JIA) or juvenile rheumatoid arthritis (JRA) and/or diagnosis of RA before the age of 16
DAS28 $> 5.1$ if biologics drugs being administrated	Any surgical procedure, including bone/joint surgery/synovectomy (including joint fusion or replacement) within 12 weeks before included in the study or scheduled to be administrated during study or Intra-articular or parenteral glucocorticoids within 4 weeks before commencement of the study
Treatment with DMARD for $\geq 3$ months and stable dose for $\geq 2$ months	Significant concomitant disease, which would impede patient participation in the investigators opinion
Generalized moderate to severe chronic periodontitis as evidenced by pocketing with clinical attachment loss (CAL $\geq 4$ mm on at least 2 non-adjacent teeth AND cumulative probing depth $\geq 40$ mm). Cumulative pocket depth is the sum of the deepest probing depths of at least 4 mm on each tooth	Periodontal treatment received within 12 months of the inclusion in the study or other dental problems contraindicate the participation in the study

#### 2.1.1.5. Randomization

Patients that qualified for the intervention study according to the inclusion/exclusion criteria were offered a verbal and written explanation of the study and a consent form (Patient Information Sheet -Treatment, and Informed Consent Form - Treatment). If willing, patients were randomized into one of the two treatment arms, stratified by their ACPA status. Randomization was performed through the Birmingham Clinical Trials Unit, which used a web-based system developed by the unit. Investigators were blinded from the group allocation for the duration of trial.

#### 2.1.1.6. Intervention and Control group

Patients allocated in the intervention group received, within 2 weeks of baseline, 2-4 sessions of non-surgical periodontal treatment by a qualified dental hygienist from the Periodontal Department of Birmingham Dental Hospital. Using ultrasonic scalers and curette instruments, this treatment (scaling and root surface debridement) aims to reduce or eliminate the pathogenic subgingival flora.

Patients allocated in the control group received oral hygiene instructions by a competent and trained NHS dental hygienist (same operator for all subjects throughout the trial). At the end of the trial, they were offered the same periodontal treatment as that received by the intervention group. If during the study, a patient

suffered an increase of more than 2 mm of CAL in one site, they received site-specific rescue treatment (scaling and root planning of the specific pocket).

All patients were reviewed after 3 and 6 months of visiting the hygienist. During this review appointments, periodontal and RA data were collected together with biological samples by the same investigator and following the same methods as in the screening visit. All data was collected and stored using an internal database at School of Dentistry.

#### 2.1.2. Systemically healthy volunteers

Approval for this study was obtained from the South Birmingham Research Ethics Committee, UK (Approval codes: Inspired - 15/WM/0006) and the study was funded by the NIHR (Grant Reference Number: DRF-2014-07-109). Biological samples from systemically healthy volunteers were obtained as part of the INSPIRED feasibility study (Influence of Successful Periodontal Intervention on Renal and Vascular Systems in patients with Chronic Kidney Disease).

##### 2.1.2.1. Recruitment of systemically healthy volunteers

A total of 40 systemically healthy patients were recruited as part of the INSPIRED feasibility study from the periodontal and oral surgery department of Birmingham Dental Hospital as well as non-dentist/hygienist staff from within the hospital.

#### 2.1.2.2. Screening appointment: clinical assessment

The screening appointment started with a first assessment of the general health in a detailed medical questionnaire and by taking the blood pressure, weight and height. Covariate data was collected at baseline such as age, gender, ethnicity, socio-economic status, education, marital status, smoking habits, alcohol consumption, comorbidities and medication.

For the assessment of periodontal status, a Periodontal Pocket Chart was performed recording the clinical attachment level, bleeding on probing, pocket depth and gingival recession.

#### 2.1.2.3. Sample collection

During the screening appointment, if patient accepted, samples of bloods, saliva, gingival crevicular fluid (GCF) and subgingival plaque were collected. However, for this thesis, only serum and subgingival plaque were analyzed.

Blood samples were collected using the same method described in section 2.1.1.3.3

Subgingival plaque was collected upon careful removal of supragingival plaque using a cotton pellet. Plaque collection was performed inserting for 10 seconds 4 endodontic paper points of size 40 per site. This procedure was repeated in 6 representative sites, one per sextant as distant as possible. The 6 selected sites

in periodontitis patients were from 3 deep sites (>3 mm) and 3 shallow sites (<3mm). If patients had healthy periodontium 3 shallow sites were selected. The total plaque collected per patients was pooled into 2 ml tubes containing tris buffer. These sites were annotated to ensure consistency if plaque was collected in future visits. Although the plaque collection methodologies were different between the two trials, it has been previously described that these methods have a good agreement and both can be used (Jervøe-Storm, AlAhdab et al. 2007)

#### 2.1.2.4. Inclusion/exclusion criteria

To participate in the systemically healthy group (for both periodontitis and periodontally healthy groups), patients were excluded if they suffered from any self-reported systemic illness including hypertension or diabetes or if they had received periodontal treatment within the last year.

-Periodontitis group: the inclusion criteria was to be diagnosed with moderate to severe periodontitis, defined as Clinical attachment level (CAL) >4mm in at least 2 non-adjacent teeth and cumulative probing depth>40 mm. Cumulative pocket depth is the sum of the deepest probing depths of at least 4mm on each tooth.

-Periodontally healthy group: the specific exclusion criteria for periodontal health was probing depth of >4mm at any site.

#### 2.1.3. Definition of periodontitis in all study participants

The classification criteria used for periodontal status was a modification of Eke&Page 2007 case definition (Table 2.2). To classify patients as periodontally healthy, a more strict criteria was used (no more than 4 sites with 4mm PD, no pockets with 5mm CAL). Clinical characteristics of patients are described in section 3.

**Table 2. 2 Modification of Eke & Page 2007 clinical case definition for periodontitis.** Moderate, mild and moderate periodontitis were classified following the author's recommendation. Healthy status were specified in patients with no more than 4 sites with 4mm PPD and no pockets with 5mm CAL. PD: pocket depth; CAL: clinical attachment loss.

Periodontal status	PPD	CAL
Health	<= 4 sites with <= 4 mm	AND < 2 sites with <=4 mm
Mild	Nor health, Nor moderate/severe	Nor health, Nor moderate/severe
Moderate	>= 2 sites with >= 5 mm	OR >= 2 sites with >= 4 mm
Severe	>= 2 sites with >= 6 mm	AND >= 1 sites with >= 4 mm



## 2.2. Antibodies to bacterial and citrullinated human proteins analysis

Based on the findings from the study of de Pablo et al., 8 citrullinated and non-citrullinated human proteins were analyzed by Enzyme-linked immunosorbent assay (ELISA) to investigate antibody production in RA and its correlation with periodontitis. These antibodies were measured in serum collected from RA patients periodontally healthy and patients with periodontitis before and after periodontal treatment, and compared to the systemically healthy control groups with and without periodontitis.

### 2.2.1. Biological sample used

Sera from patients diagnosed with Rheumatoid Arthritis (RA) and periodontitis and the appropriate controls were analyzed using Enzyme Linked Immunosorbent Assay (ELISA) to quantify the antibody response to citrullinated peptides (samples=205, patients 146).

### 2.2.2. Study population

For the baseline group comparison, sera from 87 patients diagnosed with RA (from the OPERA trial) was used to performed antibody ELISAs. Longitudinal samples of patients with RA and periodontitis (visit 1 n=42, visit 2 n=42), were

also analyzed to evaluate the effect of periodontal therapy in the auto-antibody production in RA patients.

Samples from systemically healthy controls were acquired through the INSPIRED trial and serum was available from 17 systemically healthy with periodontitis and 20 systemically and periodontally healthy controls (Figure 2. 1).

### 2.2.3. Detection of antibody to human proteins by ELISA

To measure ACPA levels, 5 human peptides in citrullinated and arginated form were used (Table 2. 3: **Peptides used for the detection of human antibodies to citrullinated proteins in serum.** ): Fibrinogen (C-Fib and Fib), Vimentin (C-Vim and Vim), Enolase 1 (CEP) and Tenascin 5 epitope (C-TNC5, TNC5). The peptides used for the assay were provided by our collaborators in Dr Venables group (Kennedy institute of rheumatology, Oxford, UK) and previously analysed by our group (de Pablo, Dietrich et al. 2014). Sequences of the reactive epitopes have been previously described (Montgomery, Venables et al. 2013).

Table 2. 3: **Peptides used for the detection of human antibodies to citrullinated proteins in serum.** X represent the citrulline residue. Data acquired from collaborators Montgomery et. al, 2013 (Montgomery, Venables et al. 2013).

<b>Antigen</b>	<b>Sensitivity</b>	<b>Reactive epitope</b>	<b>Stock concentration</b>
<b>Fibrinogen</b>	66%	<sup>36</sup> GPXVVEXHQSACKDS <sup>50</sup>	10 mg/ml
<b>Vimentin</b>	40%	<sup>59</sup> VYATXSSAVXLXSSVP <sup>74</sup>	10 mg/ml
<b>α-Enolase</b>	50%	<sup>5</sup> KIHAXEIFDSXGNPTVE <sup>21</sup>	5 mg/ml
<b>Tenascin5</b>	47%	EHSIQFAEMKXPSNFXNLEGXXKR	10 mg/ml

#### 2.2.4. Identification of standard controls for ELISA protocols

Preliminary experiments were necessary to find appropriate positive controls from sera patients to use in all plates. For CEP and Vim, pooled samples from a past cohort acquired by our collaborators was used as positive controls. Also, some patients' sera served as standards to create calibration curves for C-Vim, C-Fib, C-TNC5, Vim and CEP (Table 2. 4). Dilutions from C-Vim, C-Fib, Vim and CEP started at 1:50 and followed 8 2-fold dilutions to 1:3200 whereas dilutions for C-TNC5 started at 1:100 to end at 1:6400. However, no appropriate control was found for arginated enolase, fibrinogen and tenascin, as no patients had a strong enough positive result to the antibody ELISA. Once selected, the standards were run on each plate along with the patients samples, leaving space for a blank that only contained RIA buffer.

Table 2. 4: **Standard dilutions for antibody ELISAs.** Methods explained in section 2.2.3.1. Abbreviations: C-, citrullinated; Vim, Vimentin; Fib, fibrinogen; TNC,5 tenascin 5; CEP, citrullinated human enolase.

Standard	Antigenic peptide				
	C-Vim	Vim	C-Fib	CEP	C-TNC5
1	1/50	1/50	1/50	1/50	1/100
2	1/100	1/100	1/100	1/100	1/200
3	1/200	1/200	1/200	1/200	1/400
4	1/400	1/400	1/400	1/400	1/800
5	1/800	1/800	1/800	1/800	1/1600
6	1/1600	1/1600	1/1600	1/1600	1/3200
7	1/3200	1/3200	1/3200	1/3200	1/6400
Type of standard	Pt 1087 V1	Collaborators (Raz 29)	Pt 1117	Collaborators (Raz 18)	Pt 1158 V1

### 2.2.5. ELISA methods

The stock concentration of the peptides (donated by our collaborators from the Kennedy Institute of Rheumatology) was 10 mg/ml for all analytes except for REP and CEP, which were at 5 mg/ml. Coating peptides were diluted to a final concentration of 10 ug/ml with Coating Buffer (detailed in buffers and reagents section) and added to a 96 well plate (100 ul/well). After an overnight coating at 4° C on a rocking platform (Biometra, Germany), plates were washed 4 times with wash buffer (1xPBS, 0.05 % Tween-20) and peptides were blocked with a freshly prepared dilution of 1x Phosphate-buffered Saline (described in section Buffers and reagents) with 2% Bovine Serum Albumin for 2 hours at room temperature (RT) on a rocking platform.

After removing (not washing) the blocking buffer, sera diluted 1:100 in RIA buffer and the appropriate standard dilutions (Table 2. 4) and positive controls were incubated for 1.5 hours at RT on rocking platform. After 4 washes, secondary antibody was added (diluted 1:3000 in RIA buffer) and incubated for 1 hour at RT on a rocking platform. After another 4 washes, 100 ul/well of mixed TMB Substrate (Thermo scientific) was incubated for 5 minutes at RT immediately followed by 100 ul/well of Stop Solution (6% Sulphuric acid). Plates were read at 450 nm by a spectrophotometer (Infinite® 200 PRO, Tecan Trading AG, Switzerland). This process followed a trusted, standard protocol developed by Dr

AM Quirke (Oxford University) (Quirke, Lugli et al. 2014) (de Pablo, Dietrich et al. 2014).

#### 2.2.6. Buffers and reagents for ELISA assay

##### 2.2.6.1. RIA buffer

RIA buffer was prepared by adding 10ml of Triton-X100 into a measuring cylinder containing 500ml of distillate water, to get a final concentration of 1%. Once stirred on a magnetic plate until fully dissolved, the rest of the reagents were added and topped with 500ml of water. For a final volume of 1L of buffer, the rest of the reagents were added: Bovine Serum Albumin (BSA)(10g) , 10ml of 1M trizma hydrochloride (previously prepared by dissolving 15.7 g into 100ml of dd water at pH 7.6), sodium chloride (20.4g), 1M 2-Amino-2-hydroxymethylpropane-1,3-diol (10 ml), Triton-X 100 (10ml), sodium-deoxycholate (5 g) and 10 ml of 10% sodium dodecyl sulphate (previously prepared by diluting 1g in 10ml of water). This buffer was then stirred (Corning® PC-420D, USA) at RT until dissolved for 2 hours and stored at room temperature for a maximum of 3 months.

##### 2.2.6.2. Coating buffer

Coating buffer was prepared using 1 L of distilled water adding 1.59 g of sodium carbonate, 2.93 g of sodium hydrogen carbonate and 0.19 g of sodium azide. This buffer was then stirred (Corning® PC-420D, USA) at RT until dissolved for 2 hours and stored at room temperature.

#### 2.2.6.3. PBS buffer

Phosphate-buffered saline (PBS) used for the wash buffer and blocking buffer was stored in a 10x concentrate stored at room temperature and prepared dissolving in 1L of distilled water in 80 g of sodium chloride, 2g of potassium chloride, 14.2 g of sodium hydrogen phosphate and 2.4 g of potassium dihydrogen phosphate at pH of 7.4. PBS was stored at room temperature for a maximum of 1 month.

#### 2.2.6.4. Wash buffer

Wash buffer was freshly prepared every day, dissolving 0.5 ml of Tween-20 in 1L of PBS.

#### 2.2.6.5. Blocking buffer

Blocking buffer was freshly prepared every day, dissolving 2g of BSA in 100ml of PBS.

#### 2.2.6.6. Stop solution

Stop solution was prepared by adding 27.8 ml of sulphuric acid (stock concentration of 98%) into 500ml of distilled water. This solution was stored at room temperature for 4 months.

#### 2.2.7. Anti CCP kit assay

To measure Cyclic Citrullinated Peptides antibodies we performed the EDIA™ anti CCP assay offered by Euro Diagnostica, based on highly purified synthetic

peptides containing citrulline residues. This kit is based on an ELISA method: 96 well plates were coated with the citrullinated cyclic peptide (antigen) and diluted patients sera (1:1000) was added to the wells. After incubation for 1 hour at room temperature, unbond materials were washed away and alkaline phosphatase-labelled goat polyclonal antibody to human IgG were added and incubated for 30 minutes at RT, followed by a second washing step and incubation with a chromogenic substrate (phenolphthalein monophosphate) that result in the development of color, proportional to the quantity of serum antibody in your sample and measured at 450nm by a photo spectrometer.

#### 2.2.8. Detection of antibodies to *P. gingivalis* -related antigens

To measure human antibodies against *P. gingivalis* -antigens, 5 peptide were used (Table 2. 5): whole PPAD and immunodominant epitope 3 (citrullinated and arginated), gingipain (RgpB) and *P. gingivalis* -enolase. The peptides used for the assay were provided by our collaborators in Prof Venables group (Kennedy Institute of Rheumatology, Oxford, UK) that were previously used in studies (Quirke, Lugli et al. 2014) with the exception of RgpB that came from Prof Jan Potempa (Krakow, Poland).



Table 2. 5: Peptides used for the detection of antibodies to *Porphyromonas gingivalis* (PG) -related antigens. Abbreviations: PPAD, *P. gingivalis* -peptidyl arginine deiminase; CPP3, citrullinated immunodominant epitope 3 (RPP3, arginated); RgpB, gingipain .

Peptide	Sequence	Stock concentration	Coating concentration	Supplier
<b>PPAD</b>	CATATC-GGTACC-TGAAAAAGCTTTTACAGGCTAAA GCCTTGATTC	7 mg/ml	10 µg/ml	Oxford University
<b>CPP3</b>	CAKTDSYWT-Cit-DYTGWTFAMYDC	5 mg/ml	10 µg/ml	Oxford University
<b>RgpB</b>	RgpB-6xHis	0.81 mg/ml	5 µg/ml	Krakow institute
<b>PG-enolase</b>	ckiig-X-eilds-X-gnptvec	1 mg/ml	10 µg/ml	Oxford University

### 2.2.9. Identification of standard controls for ELISA protocols

Preliminary experiments were necessary to find appropriate positive controls from patients' sera to use in all plates. In addition, some patients' sera served as standards to create calibration curves (Table 2. 5). However, due to the low antibody response found for the anti-RPP3 and PPAD no standard was found to calculate a curve (Appendix figures).

### 2.2.10. ELISA methods and reagents

ELISA methods are described in section 2.2.5, using the appropriate peptides (Table 2. 6) to coat the plates for the identification of the antibodies. Same buffers and reagents from section 2.2.6 were used for the antibody ELISAs to *P. gingivalis* -related antigens.

Table 2. 6 Standard concentrations for antibody ELISAs to *P. gingivalis* -related antigens. Abbreviations: PPAD, *P. gingivalis* -peptidyl arginine deiminase; CPP3, citrullinated immunodominant epitope 3 (RPP3, arginated); RgpB, gingipain.

Standard	Antigenic peptide	
	RgpB	CPP3
1	1/75	1/100
2	1/150	1/200
3	1/300	1/400
4	1/600	1/800
5	1/1200	1/1600
6	1/2400	1/3200
7	1/4800	1/6400
Type of standard	Patient 1295	Patient 1115

## 2.3. Antibodies to membrane proteins from *Porphyromonas gingivalis*, *Fusobacterium Nucleatum* and *Tannerella Forsythia* analysis

### 2.3.1. Biological samples and study volunteers

Serum samples from systemically healthy controls and a subgroup of the OPERA trial were aliquoted (100 µl) and sent to the Nebraska immunology laboratory in 96 well plates that contained anonymized samples from all groups in each plate to assure consistency.

### 2.3.2. Detection of antibodies against bacterial proteins by ELISA

A collaboration with the University of Nebraska medical Centre (Dr Ted Mikuls) was arranged to investigate this novel technique to semi-quantify serum antibodies to bacterial proteins that the experimental immunology laboratory has developed. After a Material Transfer Agreement was created, serum samples were organized in 96 well plates and sent in dry-ice to Nebraska. No patient data was sent to our collaborators to assure blindness. Outer Membrane Antigens were prepared by collaborators in the Nebraska immunology laboratory, using a broth bacterial culture containing the bacteria of interest (*Porphyromonas gingivalis*, *Fusobacterium Nucleatum* and *Tanerella Forsythia*). These processes have been previously described (Mikuls, Payne et al. 2014).

## 2.4. Effect of periodontal therapy in the cytokine response in RA

### 2.4.1. Multiplex assay for cytokine quantification

Gingival crevicular fluid (GCF) was analyzed for the semi-quantification of inflammatory cytokines (sample collection explained in section 2.1.1.3.1). Once PerioPaper strips were thawed, all 4 strips from each patient were pooled into a single cryotube and eluted by adding 200 µl of elution buffer containing BSA 0.5% and 1x PBS and incubated for 1 hour at room temperature. 200 µl of the eluted samples were then aliquoted into 96 well masterplates.

Cytokine analysis was performed using a commercially available multiplex bead-based assay designed to quantitate multiple cytokines (Bio-Plex Pro™ Human Cytokine Panel, 27-Plex from Bio-Rad™ Platform) following manufacturer's instructions (Table 2. 7). A panel containing 27 cytokines was selected (Table 2. 7).

Magnetic beads were diluted to a 1x concentration in Bio-Plex assay buffer and were vortexed for 30 seconds. After pre-wetting the plate with Bio-Plex buffer (100µl), beads (50µl) were added into each well and incubated for 30 minutes at RT.

To wash the plates, wash buffer (150 µl) was added to each well and plates were attached in the magnetic holder for 30 seconds. After flicking out the liquid, plates were detached from the magnetic holder and wash buffer was again added. This process was repeated 3 times in every wash step.

Standards were prepared as 1 in 8 dilutions. Samples and standards (50 µl) were added in the plates and incubated for 30 minutes, covering the plate with sealing

aluminum foil at RT on a shaker (Luckham R11/TW, speed 2). After repeating the washing step, detection antibody (25  $\mu$ l) was added in each well in a 1x concentration (diluted in detection antibody diluent) and incubated for 30 minutes, covering the plate with sealing aluminum foil at RT on a shaker.

After repeating the wash step, streptavidin-phycoerythrin reporter (50  $\mu$ l) was added and incubated for 10 minutes. After a final wash step, beads were suspended in assay buffer (125  $\mu$ l) and shaken for 30 seconds. Fluorescence was detected on a Luminex 100/200 (USA).

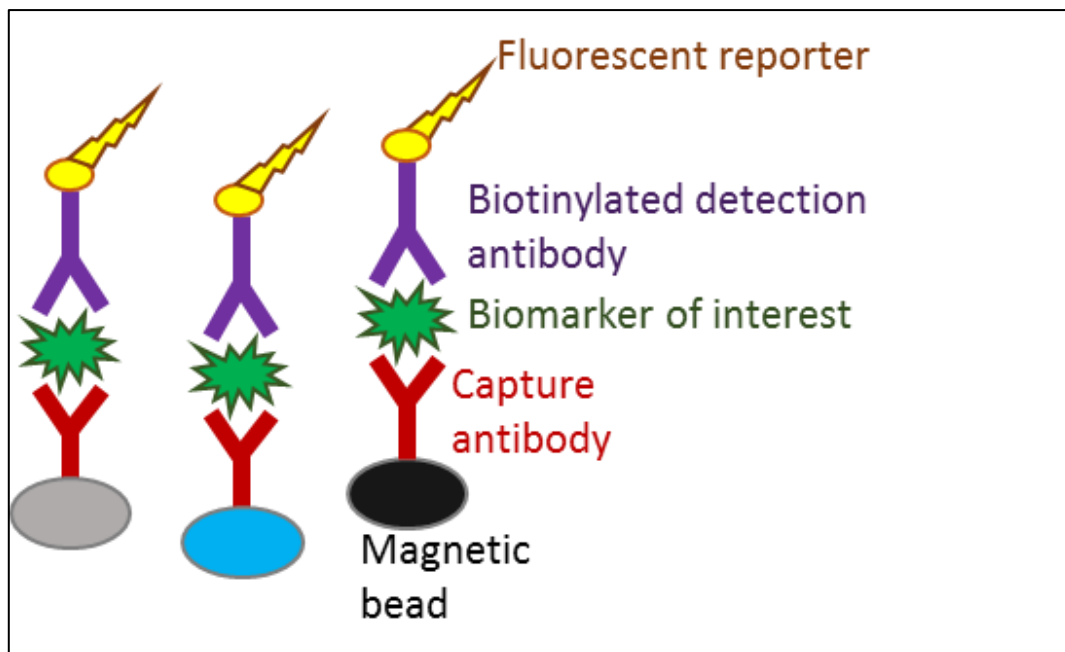


Figure 2. 3 General overview of the Bio-Plex Pro™ Human Cytokine Panel assay for multiple cytokine quantification. Magnetic beads are used to indicate where the biomarker is found and the fluorescent reporter intensity indicates the quantity.

Table 2. 7 Cytokines analyzed with the Bio-Plex Pro™ Human Cytokine Panel assay. Abbreviations: IL, interleukin; FGF, Fibroblast growth factor; GM-CSF, Granulocyte-macrophage colony-stimulating factor; G-CSF, Granulocyte colony-stimulating factor; MIP, Macrophage inflammatory protein; RANTES, regulated upon activation, normal T cell expressed and secreted; TNF, tumour necrosis factor; PDGF-BB, Platelet-Derived Growth Factor-BB ; MCP, Monocyte chemoattractant protein.

Analyte	Bead location	Analyte	Bead location	Analyte	Bead location
IL-1 $\beta$	39	IL-10	56	IFN- $\gamma$	21
IL-1ra	25	IL-12 (p70)	75	IP-10	48
IL-2	38	IL-13	51	MCP-1 (MCAF)	53
IL-4	52	IL-15	73	MIP-1 $\alpha$	55
IL-5	33	IL-17A	76	MIP-1 $\beta$	18
IL-6	19	Eotaxin	43	PDGF-BB	47
IL-7	74	FGF basic	44	RANTES	37
IL-8	54	G-CSF	57	TNF	36
IL-9	77	GM-CSF	34	VEGFs	45

## 2.5. Oral microbiome analysis in rheumatoid arthritis

### 2.5.1. Biological samples used

Subgingival plaque samples were collected, after removal of supragingival plaque with cotton pellet, from the 6 representative sites selected, one site per sextant, as distant as possible from each other (methods of sample collection explained in section 2.1.1.3 and 2.1.2.3). The method of collection in the systemically healthy group was inserting an endodontic paper point in the pocket and for the RA group was using the appropriate Gracey curette (described in section 2.1.1.3 and 2.1.2.3). Plaque samples from each patient were pooled together and stored in a cryotube containing Tris buffer and stored at -80°C until sent to Ohio State University in dry ice for the analysis of the samples.

### 2.5.2. Study volunteers

Subgingival plaque samples (n=281) were sequenced from the OPERA and INSPIRED participants according to availability of the subgingival plaque sample. 17 patients were discarded due to drop-outs or not enough clinical data available, resulting in a total number of patients included of 264 (Figure 2. 1 Study population included for the different studies of this thesis. Plaque, and serum samples were collected (methods described in section 2.1) for the analysis of the immunological and microbiological relationship between RA and periodontitis. When samples were available, the same patients were investigated from each baseline group, which overlapped in the majority of the experiments. Patients diagnosed with mild periodontitis are not represented in this diagram and are



excluded for baseline comparisons. Abbreviations: N, number of patients; RA, rheumatoid arthritis; PD, periodontitis. ELISA, enzyme-linked immunosorbent assay; OMA, outer-membrane antigen; PG, *Porphyromonas gingivalis*. Subgingival samples from two types of sites (deep pockets and shallow pockets) were selected from each patient in the systemically healthy with periodontitis group (n=17) to compare the two locations. To investigate the effect of periodontal therapy in RA, plaque samples were collected from the two randomized groups (intervention and control) after 3 and 6 months after randomization (n=240)

#### 2.5.3. DNA isolation

Bacterial DNA was extracted from the plaque samples in the research laboratories of the Ohio State University School of Dentistry. Plaque samples collected with currettes were diluted in PBS (200µl) and centrifuged (8000 rpm for 1 minute) to remove the Tris-buffered saline and other salts. Plaque samples were collected with paper-points, 200 µl of the solution was added to the vial containing 0.25 g glass bead and beat for 60 seconds at 500 rpm twice followed by centrifuging (1400 rpm) for 2 minutes. Supernatant was carefully aspirated and placed in a labelled sterile 1.5 ml tube.

Bacterial DNA was isolated using Qiagen DNA MiniAmp® kit (Qiagen, Valencia, CA, USA) and protocol manufacturer instructions were followed.

#### 2.5.4. Sequencing

Library preparation and sequencing (the reading of the nucleotides present in DNA) was conducted in the Mr DNA laboratories (Mrdnalab, Shallowater, TX, USA) (Figure 2. 4). PCR primers of the V1-V3 (spanning E.coli 16S gene regions 8-27 and 519-536) and V7-V9 (spanning E.coli 16S gene regions 1099-1114 and 1528-1541) of the 16S rRNA gene were used (Kumar, Brooker et al. 2011). Two sets of primers were used, since each set is capable of detecting a range of genera that the other fails to recover. Together they allow the recovery of a wider range of the microbiome than is possible with a single primer alone. As some genera were picked up by both sets of primers, to prevent overcounting, the number of sequences assigned to an OTU by both primers was reduced by half. The 16s amplicons were quantified using the Quant-iT PicoGreen dsDNA reagent and kit (Invitrogen). Equimolar concentrations of each amplicon were pooled and sequenced on the HiSeq 2500 system (Illumina). Paired-end sequencing was used to sequence both ends of the fragment and generate high-quality, alignable sequence data. The sequence lengths obtained (mean +/- std) were 501.5 +/- 18.7, with a total of 29,107,462 sequences found in all 281 samples investigated and a median of 103,208 sequences per sample.

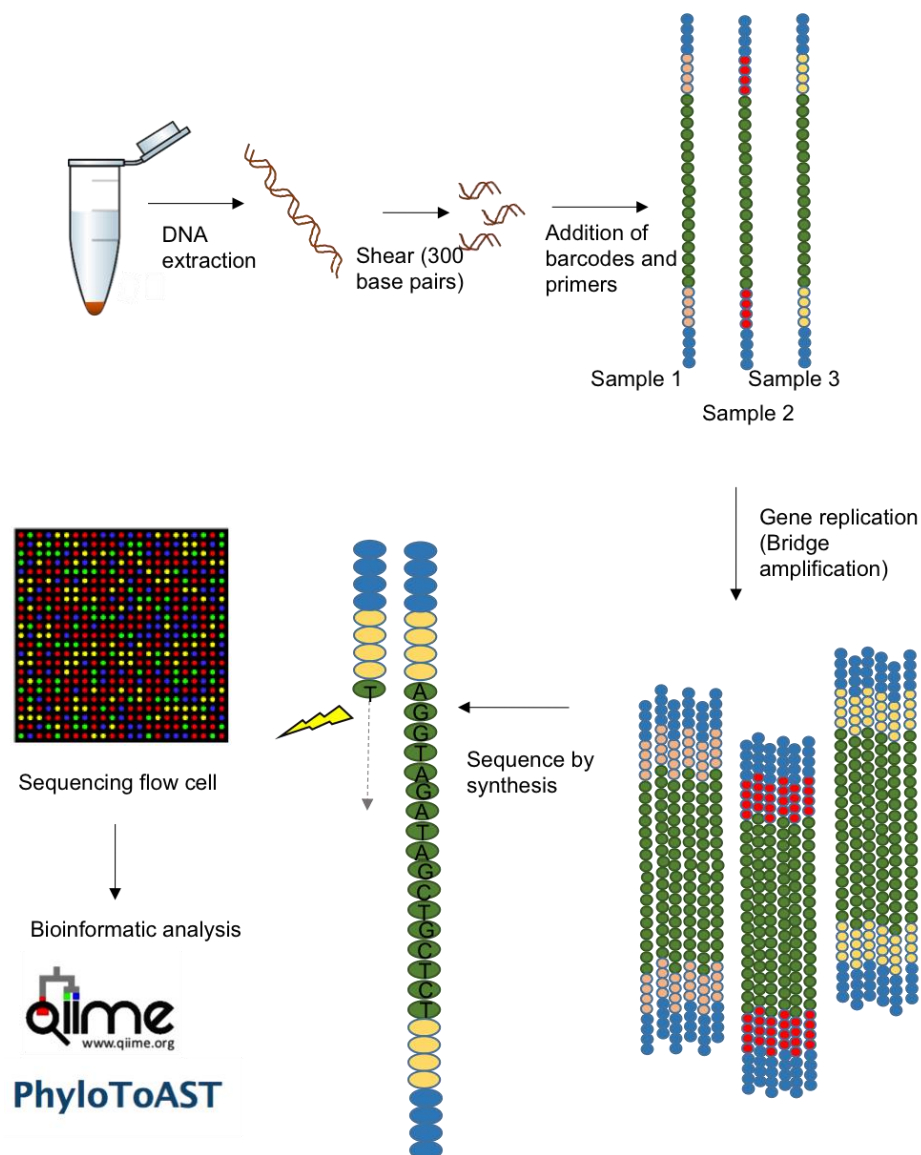


Figure 2. 4 **Workflow of 16s r-RNA analysis with Illumina MiSeq.** After extraction of DNA from the plaque samples, 16S rRNA sheared genes were tagged with unique tags and primers were added which contained Illumina adapter sequences. Genes were then amplified via PCR using primers (4) complementary to the adapter sequences. Library samples were be pooled and sequenced on the MiSeq platform, incorporating a nucleotide into the strand of DNA releasing a unique fluorescent signal that generates the genetic reads. Adapted from [www.illumina.com](http://www.illumina.com).

#### 2.5.5. Sequence data pre-processing

Once the sequences of DNA have been generated, Phylogenetic Tools for Analysis of Species-level Taxa (PhyloToAST) version 1.4.0rc1 and Quantitative Insights Into Microbial Ecology (Qiime) version 1.9.1 pipelines were used to pre-process the sequences to reduce errors and bias produced in the sequencing (Figure 2. 5).

Quality control of 98% was required for adaptor sequences and sequences of less than 300bp were discarded. The sequences were binned by sample and denoised using `denoise_wrapper.py` to reduce sequencing errors. All denoised sequences were aggregated and de novo operational taxonomic units (OTUs) were identified. Chimeric sequences were depleted using ChimeraSlayer (v. 1.9.0, `identify_chimeric_seqs.py`). All quality filtered sequences were aggregated and clustered into OTU at 97% similarity using the UCLUST65 method. Sequences with an average quality score of 30 over a sliding window of 50bp and length >200 bp were assigned a taxonomic identity by alignment to the HOMD database using the Blastn algorithm at 97% identity.

An OTU was included in the analysis if it was present in more than 5% of samples, at an abundance of at least 0.001% as previously described (Mason, Preshaw et al. 2015).

After merging the two primers, the number of species assigned to an OTU by both primers was reduced by half to prevent doubling sequences picked by both primers. Primer averaging was carried out as previously described [4, 5, 6]. To

parse the OTU-sequence data, first any OTUs that occurred in less than 5% percent of samples and/or OTUs that make up less than 0.01% of the overall sequences were removed. The removed OTUs are separated in a file for future examination. For the following steps, a representative sequence was picked from the database for each OTU (instead of operating with the entire dataset). Finally, taxonomic identity was assigned to each OTU by alignment of the representative sequence to the HOMD database [7] using the Blastn algorithm.

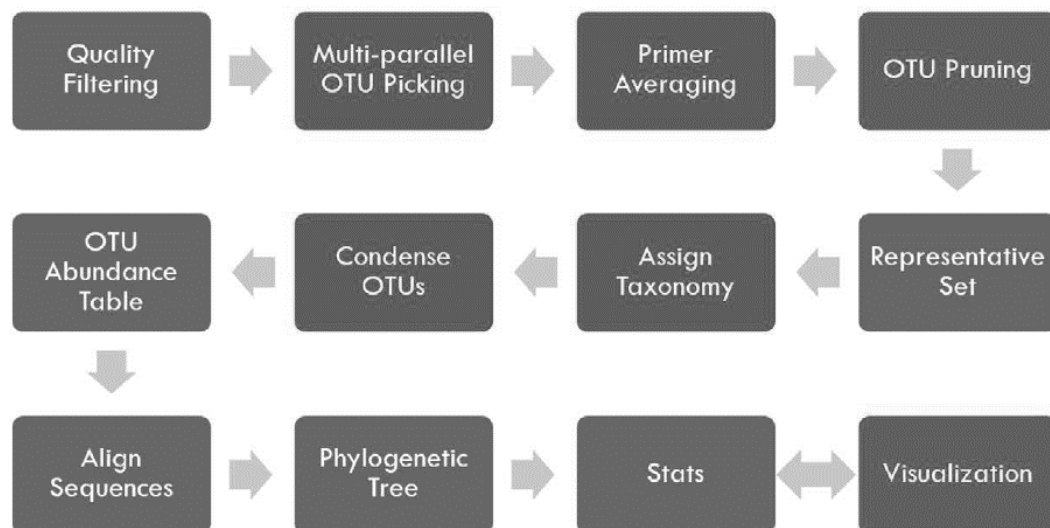


Figure 2. 5 PhyloToAST pipeline analysis process. Modified with the permission from Dabdoub et. Al (Dabdoub, Fellows et al. 2016).

#### 2.5.6. Microbiome data analysis

Rarefaction analysis was performed to confirm if saturation was reached for all the samples, using the `alpha_rarefaction.py` script (Qiime).

Beta diversity (between-group) was investigated performing Principal coordinates analysis (PCoA) using both distances matrices: phylogenetic (UniFrac weighted and unweighted) and non-phylogenetic (Bray–Curtis). Significant differences between clusters was calculated using the Adonis and ANOSIM tests. PCoA plots were generated by the python package PhyloToAST using `PCoA.py` script.

Alpha diversity (within group) was analyzed using Abundance Coverage Estimator (ACE) and Shannon diversity using `alpha_diversity.py` script, and differences between group-wise alpha diversities was measured using Mann-Whitney U test. Phylogenetic trees were created with iTOL (<http://itol.embl.de/>, version 3.4.1).

The core species in each group were identified using Qiime's script (`core_microbiome.py`) when species were present in at least 80% of the patients in each group.

Differences between species relative abundance in each group were calculated using Deseq (`differential_abundance.py`) and significance was obtained from adjusted p values for multiple comparisons (FDR-adjusted Wald Test). This

function uses a negative binomial distribution of raw counts to estimate between-group differences, while accounting for sampling effort (library size) and dispersion of each category (taxon or functional gene).

Network correlations were investigated to test for differences in co-occurrence patterns between microbial communities from different ecosystems. Significant correlations were calculated using Spearman's correlation ( $p < 0.05$  and  $\rho \geq 0.75$ ) in JMP (SAS Institute Inc.) and inserted in Python (Networkx package {Schult, 2008}) to create the graph structures. These correlations were then visualized using the web tool Gephi (<https://gephi.org/>). Network anchor OTUs were defined as significant different abundance between the 2 groups, high betweenness centrality (top 20%) and belonging to the core microbiome of each group.

## 2.6. Statistical analysis

### 2.6.1. Clinical data

Clinical results to describe the study population were presented as median and interquartile range (IQR) for continuous data and proportions for categorical data

### 2.6.2. Raw data

Calibration curves were interpolated from Optical Density Concentrations to calculate the concentration of antibody levels, which were presented in Arbitrary Units per ml (AU/ml) when standard dilutions were available. In cases of non-available standards, results were presented in optical density units (ODs). Raw

data was processed using Graphpad Prism 7.0 (GraphPad Software Inc, California, USA). Graphs were generated with Graphpad Prism. Raw data produced by the cytokine experiments was processed using xPONENT® software (USA), which interpolated the fluorescence measure against the calibration curves for each standard to calculate the concentration (AU/ml). After interpolating the standards to calculate AU/ml, rare out of range units were found. As the amount of peptide and kits were limited to be able to dilute those samples and repeat the experiments, the raw data was used for the statistical analyses, for the out of range data.

### 2.6.3. Group comparison and correlations

Statistical tests were run using IBM Statistics SPSS 23. To test normality, Kolmogorov-Smirnov test was used. Statistical tests were run using IBM Statistics SPSS 23. Group comparisons between patients classified as Rheumatoid arthritis periodontally healthy (RA), Rheumatoid with periodontitis (RAPD), systemically healthy with periodontitis (PD) and systemically and periodontally healthy (H) were conducted using Kruskal-Wallis test and significance between each group comparison was calculated using Dunn's multiple comparison test (significance  $p < 0.05$ ).

Differences between level of antibodies at baseline (V1) and 6 months post-intervention (V2) were discovered using Wilcoxon test (significance at  $p < 0.05$ ), in



each randomization group. Significant correlations between the serum antibodies to bacterial antigens, relative abundance of these bacteria and clinical parameters, were calculated performing the Spearman's correlation test ( $p < 0.05$  and  $\rho \geq 0.75$ ) in JMP (SAS Institute Inc.). Correlations between the changes in this parameters (differences between baseline and visit 3) and between the serum concentration of cytokines and clinical parameters and its changes were investigated in the same manner.

### **3. RESULTS – CIRCULATING ACPAS IN RA AND PERIODONTITIS**

## Introduction

The hypothesis of the study presented in this chapter was that, in rheumatoid arthritis, patients with periodontitis have higher level of serum antibodies to citrullinated proteins compared to those with healthy periodontium and that, after periodontal therapy, these antibody levels are reduced.

The aims were:

- 1) To compare the level of serum antibodies to human citrullinated (and non-citrullinated) proteins in groups of patients diagnosed with RA and periodontitis and the appropriate control groups.
- 2) To analyse the effect of periodontal therapy on the level of these antibodies in RA patients compared to a control group that did not receive such therapy.

RA antigens were detected in collaboration with Prof Patrick Venables and his group in Oxford University, which had developed RA citrullinated antigens in established ELISA protocols for the semi-quantification of antibodies to citrullination.

This chapter presents the results from serum antibody ELISAs to cyclic citrullinated peptide (CCP) and 4 human (citrullinated and arginated) proteins: Fibrinogen (C-Fib and Fib), Vimentin (C-Vim and Vim), Enolase 1 (CEP and REP), and Tenascin 5 (C-TNC5 and TNC5). ELISA methods are described in detail in chapter 2.2.

### 3.1. Study volunteers

For this project, sera collected from 124 patients from the OPERA and INSPIRED cohorts (recruitment and study population explained in chapter 2) were analysed, of which 87 patients were diagnosed with RA and 37 patients were systemically healthy (Table 3. 1 Clinical characteristics of study population. Continuous data reported as median (IQR), as most of the variables followed a non-normal distribution, categorical data represented as proportions (%).

From all patients diagnosed with RA, only 24 patients (27%) were periodontally healthy, showing a majority of RA patients presenting different degrees of periodontal disease (73%). Periodontitis severity ranged from 13 patients with mild periodontitis (16% of total RA) and 50 patients (60% of total RA) with moderate/severe periodontitis according to the Eke&Page case definition (Eke, Page et al. 2012). Patients with mild periodontitis were not included in the main cross-sectional analysis. These periodontitis patients were selected from the OPERA cohort randomly in order to have variety of periodontal status, so percentages of severity of PD does not necessarily reflect the RA population (Figure 3. 1.)

A group of systemically healthy PD (moderate and severe) of 17 patients and a group of systemically healthy and periodontally healthy group of 18 patients, were

selected as controls for this study, after the exclusion of 2 patients diagnosed with mild periodontitis.

Patients with periodontitis and active RA were selected from the longitudinal part of the OPERA study (exclusion/inclusion criteria described in Chapter 2.1). Serum from a total of 42 patients were analyzed in this chapter, excluding those patients who did not have enough clinical data, dropouts or those patients that did not donate serum samples at one of the visits. The majority of patients in this group were diagnosed at baseline with moderate/severe periodontitis and only 2 patients diagnosed at baseline with mild disease. From the 42 eligible patients, 20 patients were randomly allocated in the immediate group and 22 in the delayed group. Differences in clinical parameters at baseline between immediate and delayed group were not significant (Table 3. 2).

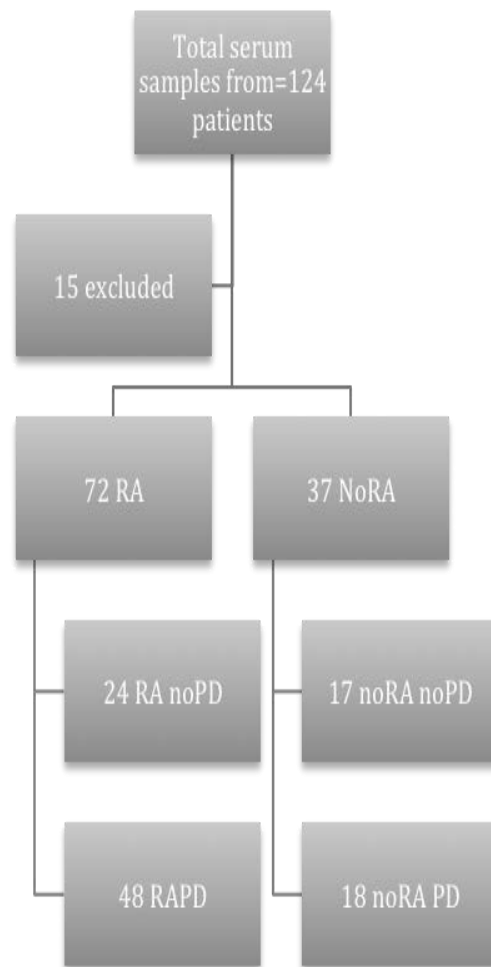


Figure 3. 1 Flow diagram of cross-sectional study population subgroup from the OPERA and INSPIRED trial (explained in section 2.1). Abbreviations: RA, rheumatoid arthritis; RA noPD, rheumatoid arthritis and healthy periodontium; RAPD, rheumatoid arthritis and periodontitis; NoRA, systemically healthy. NoRA noPD, systemically healthy with healthy periodontium; noRA PD, systemically healthy with periodontitis.

Table 3. 1 Clinical characteristics of study population. Continuous data reported as median (IQR), as most of the variables followed a non-normal distribution, categorical data represented as proportions (%). DAS28, ESR, VAS, tender and swollen joints not recorded for systemically healthy controls (“-”). Data from mild periodontitis patients excluded. Abbreviations: RA, rheumatoid arthritis; PD, chronic periodontitis; n, number of patients; CumPPD, cumulative probing depth; PPD, probing pocked depth; BoP, bleeding on probing; CAL, clinical attachment loss; DAS, disease activity score; ESR, erythrocyte sedimentation rate; VAS, visual analogue score (for activity of RA in the past week).

	RAPD (n=45)	RAnoPD (n=24)	noRAPD (n=18)	NoRA NoPD (n=19)
Female proportion	72%	71%	55.6%	63%
Ethnicity				
-Asian	14%	4%	5.6%	11%
-White	36%	49%	78%	89%
-Black	6%	0%	11%	0%
Smoker				
-current	22%	4%	5.6%	5%
-former	22%	21%	55.6%	11%
-never	48%	67%	33.3%	84%
Alcohol				
-Never	40%	45%	11%	15%
-1-4 times/month	24%	17%	16%	65%
-1-4 times/week	10%	18%	44%	15%
- >4 times/week	16%	20%	16.7%	5%
BMI	28.3 (6.6)	26.8 (7.6)	27.7 (4.1)	25.9 (5.2)
Age (years)	60(16)	61.5 (13)	48(8.5)	37(15)
CumPPD (mm)	61 (42)	12(6)	109(31)	5(12)
PPD (mm)	3.8 (0.7)	2.29 (0.4)	3.17(1)	1.56(0.3)
BoP	0.25(0.29)	0.08(0.17)	0.39(0.25)	0.02(0.11)
CAL (mm)	3.3(1.2)	2.48(1.04)	3.9(1.9)	1.7(0.28)
DAS28	4.7(2.7)	2.8(2.4)	-	-
ESR	21(30)	8(27)	-	-
VAS	60(35)	41(30)	-	-
Tender joints	9(15)	4.5(10)	-	-
Swollen joints	1(3)	0.01(3)	-	-

Table 3. 2 Clinical parameters at baseline in the two randomization groups. Values are represented as median (max, min) significant differences between visit 1, baseline (V1) and visit 2, 6 months after therapy (V2) are calculated using Mann-Whitney U test (significance <0.05) and Pearsons Chi squared test for nominal data. N=22 delayed group, 19 immediate group. Abbreviations: PPD, probing pocket depth; CAL, Clinical Attachment level; BOP, bleeding on probing; DAS 28, disease activity score 28; ESR, Erythrocyte sedimentation rate.

	<b>Delayed group baseline median (IQR)</b>	<b>Immediate group baseline median (IQR)</b>	<b>Sig two tailed p value</b>
<b>PPD (mm)</b>	3.15 (0.57)	3.2 (0.88)	0.54
<b>CAL (mm)</b>	3.42 (0.82)	3.59 (1.2)	0.36
<b>BOP</b>	0.24 (0.26)	0.29 (0.44)	0.262
<b>Das 28</b>	5.27 (1.9)	4.86 (2.4)	0.12
<b>ESR</b>	26.5 (27)	25.5 (28.5)	0.18
<b>Tender joints</b>	13.5 (12.3)	8 (13.5)	0.5
<b>Swollen joints</b>	1.5 (4)	1 (2)	0.53
<b>CRP</b>	3.9 (14.1)	3.7 (8.1)	0.77
<b>Age (years)</b>	57 (11.8)	61 (16)	0.82
<b>% Female</b>	86.4%	68.4%	0.17
<b>% Current Smokers</b>	18.2%	31.6%	0.97



### 3.2. Cross-sectional analysis

#### 3.2.1. Antibodies to citrullinated and uncitrullinated human peptides group comparison

The cyclic-citrullinated peptide (CCP) test is commonly used by rheumatologists to semi quantify overall levels of antibodies against citrullinated proteins for the classification of patients in ACPA+ and ACPA-. In this study, RA patients presented significantly higher levels of anti-CCP compared to the systemically healthy groups. In this latter group, all patients presented anti-CCP levels close to undetectable, even patients with periodontitis ( Figure 3. 2)

Antibody levels to the specific citrullinated peptides studied followed the same tendency as the observed in the anti-CCP results (Figure 3. 2, Figure 3. 3). Antibody levels to the arginated (non-citrullinated) peptides were low in all the groups, and only antibodies to Fibrinogen were non-significantly increased in the RA groups.

Previously, de Pablo et. al represented similar data classifying patients as ACPA positive or negative, if patients had ACPA levels above the 98th percentile of healthy controls. This was calculated here showing that 81% of the RA patients from both RAPD and RA noPD groups were anti-CCP positive (Figure 3. 4), From the 18 anti-CCP negative patients found in the RA group, no differences were

found between the two groups, as 24% from the RAPD group and 25% from the RA noPD group were anti-CCP negative (Pearsons Chi-squared test).

Interestingly, the percentage of ACPA-positive patients in the RAPD compare to RA group, varies for each citrullinated antigen: C-Fib and C-TNC5 show a higher percentage of positivity in the RAPD group, while C-Vim and CEP1 show higher percentage of positivity in the RA group. However, differences between proportions of ACPA + patients in the RAPD group and the RA with healthy periodontium group were not significant for all the antigens studied (Pearsons Chi-squared test).

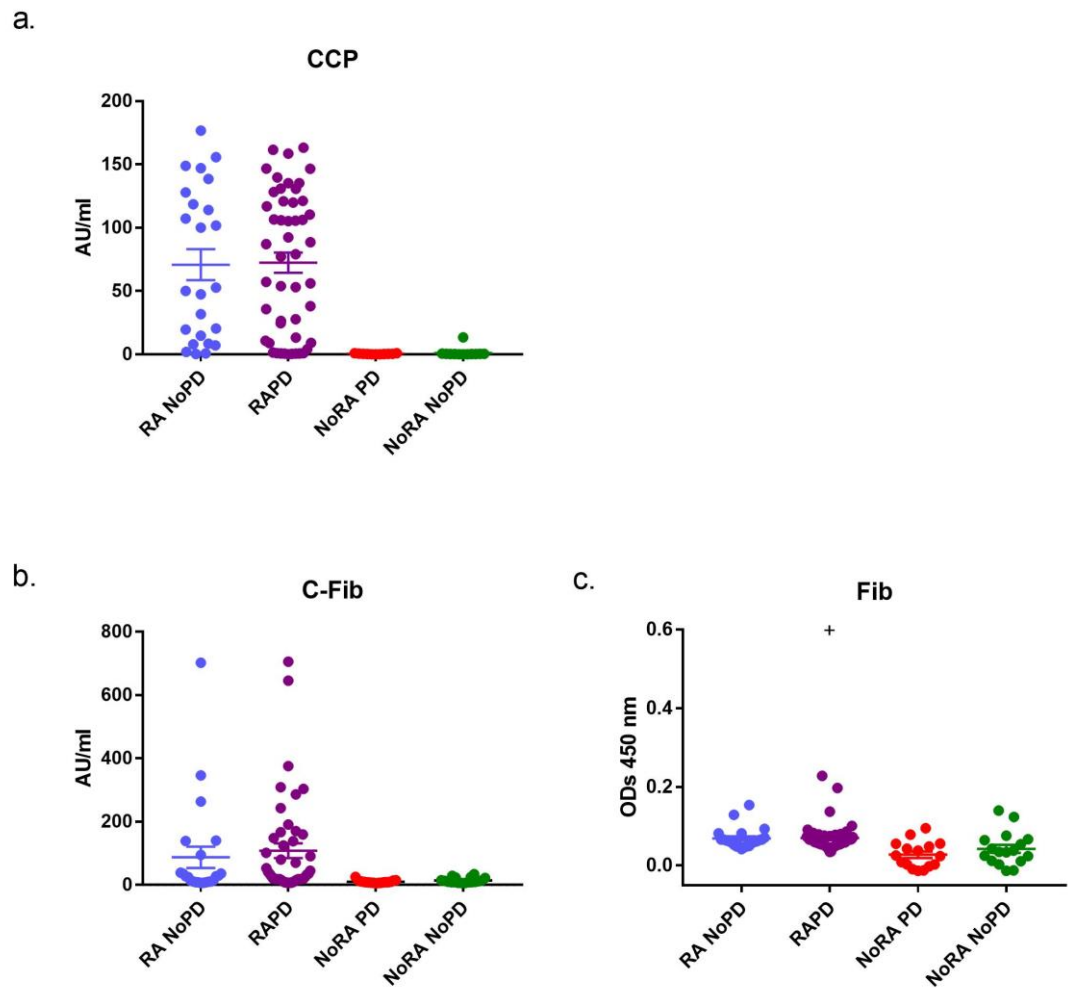


Figure 3. 2 Levels of antibodies detected in patient sera against citrullinated and arginated peptides in the different study groups: Rheumatoid arthritis with healthy periodontium (RA noPD) (n=24); baseline data from RA with Periodontitis (RAPD) (n=50); systemically healthy with periodontitis (NoRA PD) (n=17); systemically and periodontally healthy controls (NoRA NoPD) (n=18). Data represented in scatter plot graphs and coloured lines show the group mean and SEM. Each individual graph represents the results for each peptide, both citrullinated (C-) and uncitrullinated: a. Cyclic citrullinated peptide (CCP); b. and c. Fibrinogen (Fib). Significant differences (“\*\*”) between groups were analysed using the Kruskal-Wallis test and Dunn’s comparison (significant if  $p < 0.05$ ). The “+” symbol represents an outlier. Methods described in section 2.2.

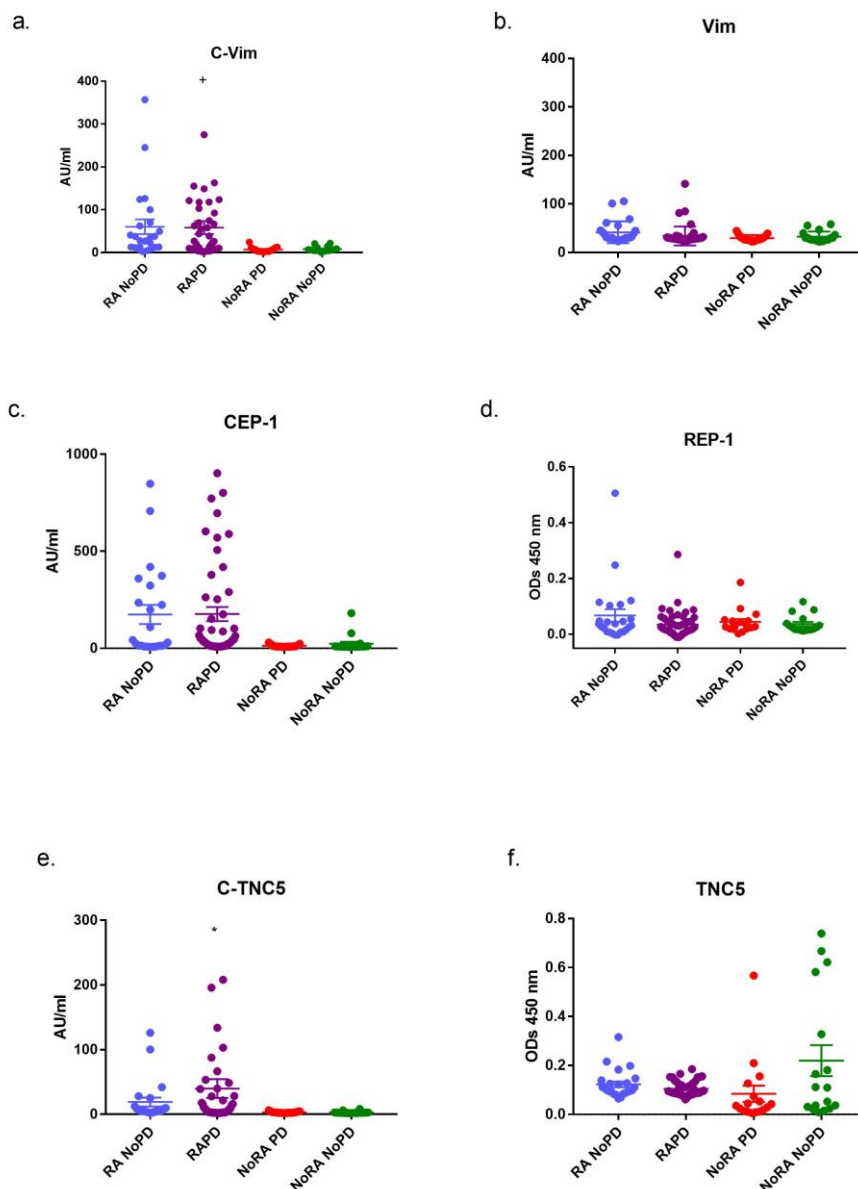


Figure 3. 3 Levels of antibodies detected in patient sera against citrullinated and arginated peptides in the different study groups: Rheumatoid arthritis with healthy periodontium (RA noPD) (n=24); baseline data from RA with Periodontitis (RAPD) (n=50); systemically healthy with periodontitis (NoRA PD) (n=17); systemically and periodontally healthy controls (NoRA NoPD) (n=18). ). Data represented in scatter plot graphs and colored lines show the group mean and SEM Each individual graph represents the results for each peptide, both citrullinated (C-) and uncitrullinated: a. and b. Vimentin (Vim); c. and d. Enolase 1 (CEP, REP); e. and f. Tenascin 5 (TNC5). Significant differences (“\*”) between groups were analysed using the Kruskal-Wallis test and Dunn’s comparison (significant if  $p < 0.05$ ). “+” symbol represents an outlier. Methods described in section 2.2.

### Percentage of patients with ACPA levels over 98 healthy percentile

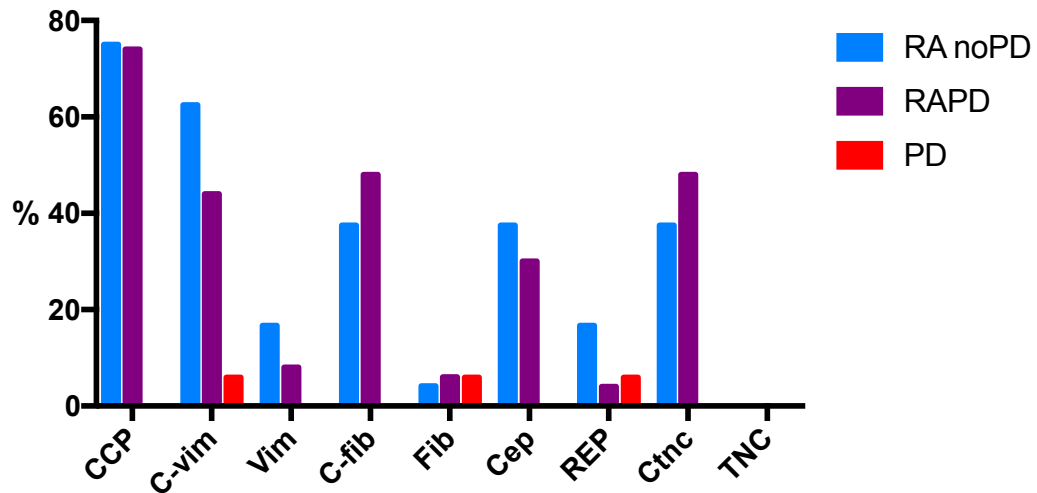


Figure 3. 4 Percentage of subjects with antibody levels above the 98<sup>th</sup> percentile of healthy controls per analyte. Analytes: citrullinated (C-) and uncitrullinated: Fibrinogen (Fib), Vimentin (Vim), Enolase 1 (CEP, REP), Tenascin 5 (TNC5), cyclic citrullinated peptide (CCP). Rheumatoid arthritis with healthy periodontium (RA noPD) (n=24); baseline data from RA with Periodontitis (RAPD) (n=50); systemically healthy with periodontitis (NoRA PD) (n=17); systemically and periodontally healthy controls (NoRA NoPD) (n=18). Significance (Pearsons Chi-squared test) if  $p < 0.05$ .

### 3.2.2. Subgroup analysis

#### 3.2.2.1. ACPA+/- comparison

As expected, in subgroup analyses stratified by ACPA status, the percentage of ACPA+ patients with antibody positivity against the rest of the citrullinated and non-citrullinated antigens was higher than in the ACPA negative group. However, there were no significant differences (Pearsons Chi-squared test) between the two RA groups with or without periodontitis, in both ACPA positive or negative stratification (Figure 3. 5).

Interestingly, while a majority of RA patients were anti-CCP positive (81%), 40% of patients that were anti-CCP negative had antibodies to C-Vim (18 patients) and most of these anti-CCP-/CVim+ patients were periodontally healthy (Figure 3. 5). This highlights the importance of testing specific ACPAs.

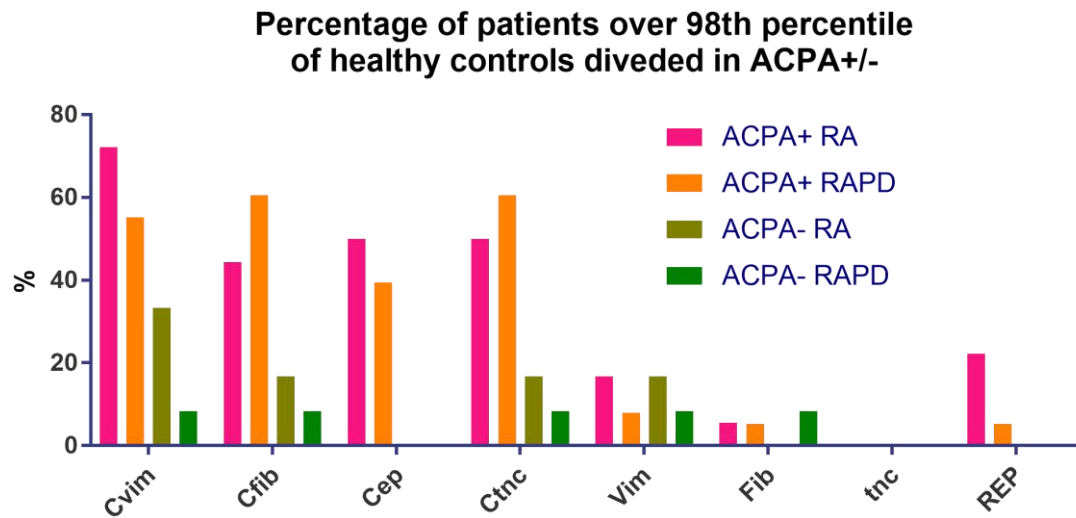


Figure 3. 5 Percentage of patients with antibody levels over the 98<sup>th</sup> percentile of healthy controls stratified in ACPA positive and negative for anti-CCP. Antibody levels tested against: both citrullinated (C-) and uncitrullinated: Fibrinogen (Fib), Vimentin (Vim), Enolase 1 (CEP, REP), Tenascin 5 (TNC5), cyclic citrullinated peptide (CCP). Significance (\*) defined as  $p < 0.05$ , test used Pearsons Chi-squared test. Abbreviations: ACPA, anti-citrullinated protein antibody; RA, rheumatoid arthritis; PD, periodontitis.

#### 3.2.2.2. Periodontal health comparison

Clear differences were found in the antibody levels to citrullinated peptides in the RA groups compared to systemically healthy controls (Figure 3. 2, Figure 3. 3) for all peptides investigated. However, the periodontal status did not seem to affect the antibody production in RA or in systemically healthy individuals. To further investigate this, all patients (RA and no-RA) were grouped according to their periodontal health (see section 2 for periodontal health definition) which showed no differences between the groups (Figure 3. 6). From all patients investigated (n=124) , 42 were classified as periodontally healthy, 15 patients as mild periodontitis and 67 as moderate to severe periodontitis. Although there are considerably less patients in the mild periodontitis group, shifting the results in the anti-CCP levels (Figure 3. 6.a), the patients with high antibody response (which represent the RA patients) are equally distributed in all groups for all analytes.



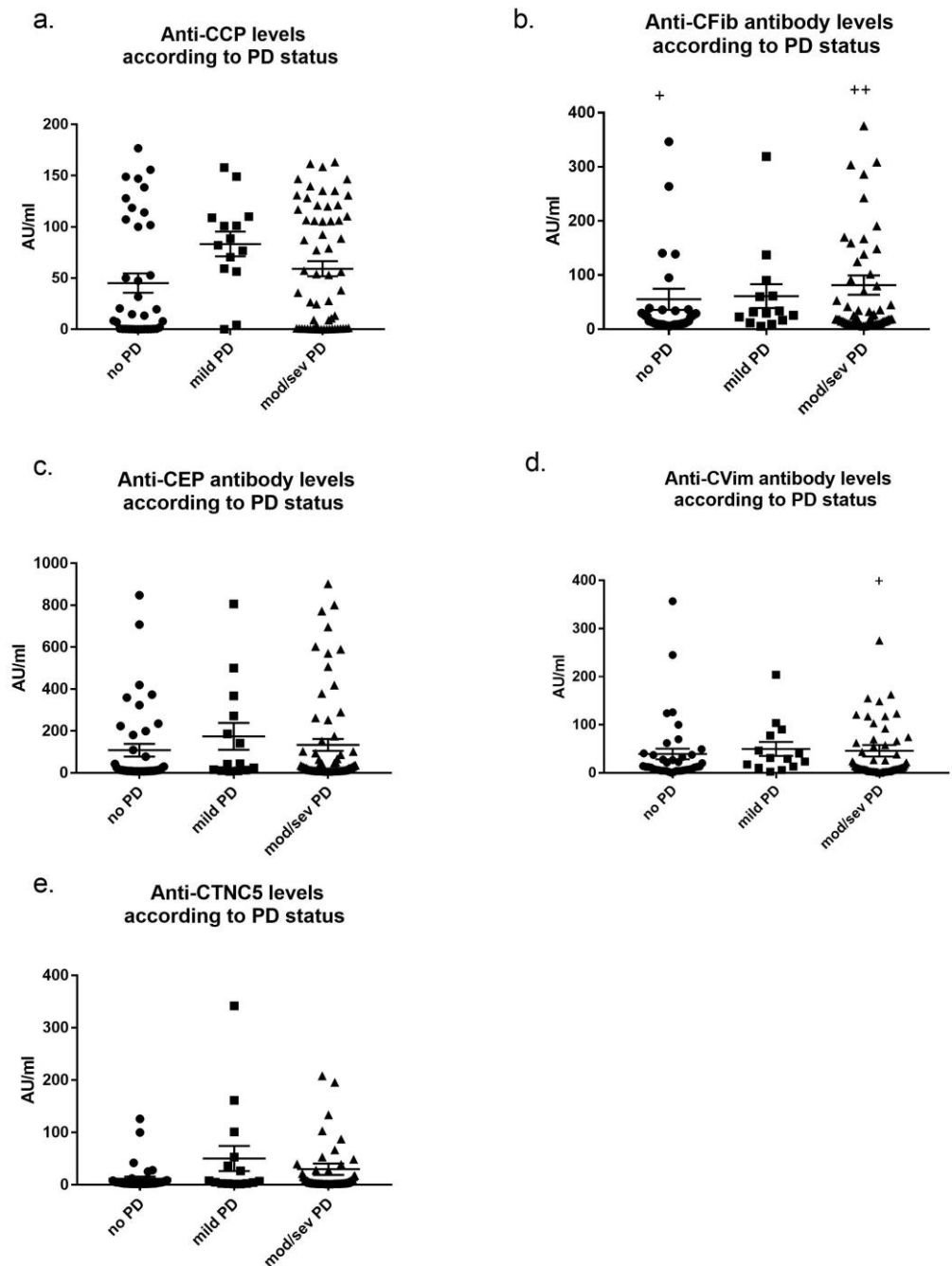


Figure 3. 6 Antibodies to citrullinated proteins according to periodontal status. Analytes investigated: a. cyclic citrullinated protein (CCP); b. citrullinated enolase (CEP); c. citrullinated Fibrinogen (C-Fib); d. citrullinated Vimentin (C-Vim); e. citrullinated tenascin 5. Differences between groups were tested using Kruskal-Wallis test with Dunn's multiple comparison (significance ("\*") =  $p < 0.05$ ). "+" symbol represents an outlier. Methods explained in section 2.

#### 3.2.2.3. Smoking comparison

A similar analysis was performed to investigate the effect of smoking and smoking history in the antibody response in all patients (Figure 3. 7). From all patients investigated (n=116; 8 patients were excluded as no smoking data was available), 16 were current smokers, 33 patients were former smokers and 67 patients had never smoked. Although no significant differences were found between the groups, an interesting similar trend was observed in the antibodies to CCP, C-Fib and CEP, with the highest mean antibodies in the smoker group, and increasing for former smoker group and with the lowest mean antibodies in the group never smoked. However, as there are considerably fewer patients in current smokers group, this could be the artefact that explains the higher mean found in this group as, in the smoker and former smoker groups with bigger number of patients, the patients with high or very low antibody response (which represent the RA patients) are similarly distributed in all groups for all analytes.

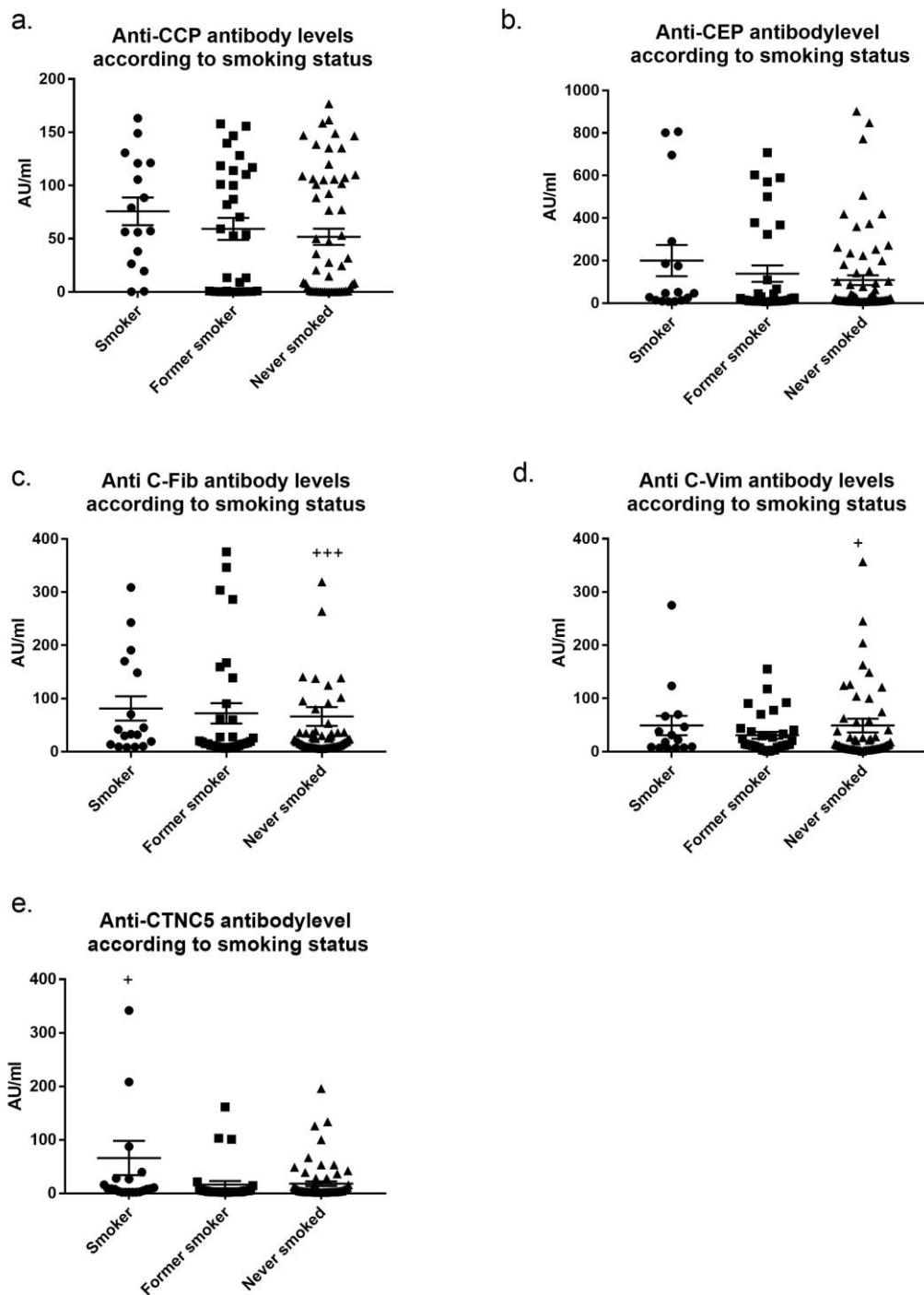


Figure 3. 7 Antibodies to citrullinated proteins according to smoking status. Analytes investigated: a. cyclic citrullinated protein (CCP); b. citrullinated enolase (CEP); c. citrullinated Fibrinogen (C-Fib); d. citrullinated Vimentin (C-Vim); e. citrullinated tenascin 5. Differences between groups were tested using Kruskal-Wallis test with Dunn's multiple comparison (significance ("\*") =  $p < 0.05$ ). "+" symbol represents an outlier. Methods explained in section 2.

### 3.2.2.1. Cross-reactivity and overlapping of antibodies to citrullinated proteins

In our study, the majority (78%) of RA patients were found to be positive to anti-CCP and positive to at least one of the citrullinated antigens investigated (Figure 3. 8). From all anti-CCP positive patients (n=68), 7 expressed antibodies to all the individual citrullinated antigens but also some of these anti-CCP positive patients were not positive for any of the citrullinated antigens studied (n=6). There did not seem to be a clear relationship with periodontal status as patients with and without periodontitis were equally distributed across all the citrullinated peptides. Furthermore, 4 of the anti-CCP negative patients expressed antibodies against at least one of the other citrullinated peptides (one patient anti-CCP negative was positive for anti-C-Fib, C-Vim and C-TNC5) but with no relationship to the presence of periodontitis. This represents 22% of the RA patients that were diagnosed as ACPA negative but who showed antibodies to specific citrullinated antigens. No clear pattern could be drawn on the cross-reactivity of the peptides in anti-CCP positive or negative patients or according to periodontal status.

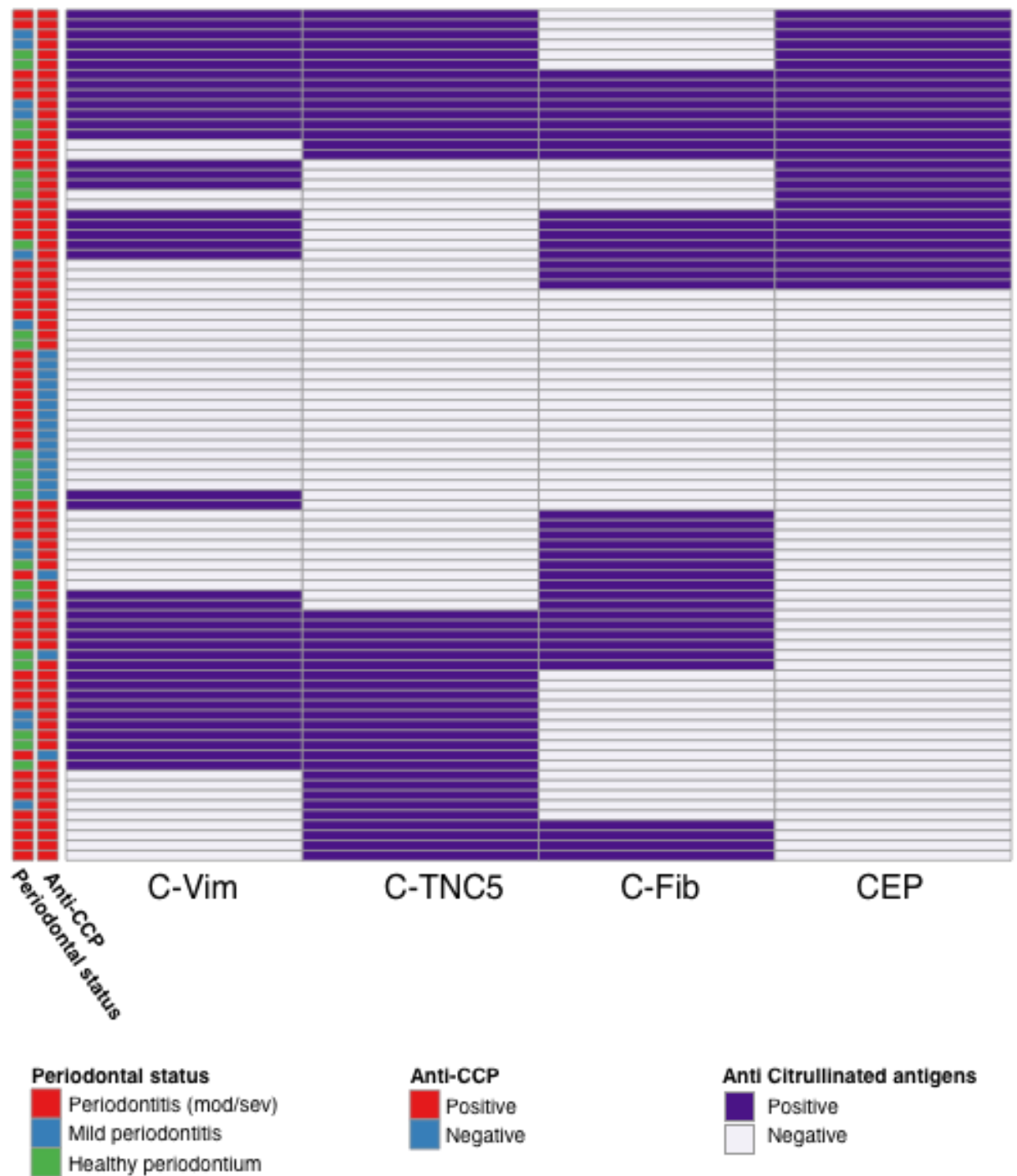


Figure 3. 8 Heatmap of antibody positivity to citrullinated epitopes in each RA patient (n=87). Patients with positive antibodies to each antigen had antibody levels over the 98<sup>th</sup> percentile of healthy controls. Graph created using a web tool ([biit.cs.ut.ee/clustvis/](http://biit.cs.ut.ee/clustvis/)) (Metsalu and Vilo 2015). Abbreviations: CCP, cyclic citrullinated peptide; C-TNC5, citrullinated tenascin; C-Fib, citrullinated fibrinogen; CEP, citrullinated enolase.

### 3.3. Longitudinal analysis

#### 3.3.1. Effect of periodontal therapy on clinical parameters

The effect of periodontal therapy on clinical parameters is represented in Table 3. 3 (n=42). A significant reduction of periodontal parameters in pocket depth and bleeding on probing was achieved after periodontal therapy. On the contrary, there was a significant increase of these parameters in the delayed group (Table 3. 4). The RA parameters, such as the Disease Activity Score (DAS 28) were significantly reduced after periodontal therapy, while the delayed group experienced a non-significant increase of this parameter. ESR and CRP were correlated at baseline (Spearman's correlation  $r=0.57$ ,  $p<0.001$ ), but these systemic markers of inflammation did not significantly decrease after 6 month of periodontal therapy. These markers, in the delayed group, both change significantly but not towards the same direction, but these changes might be due to RA flare ups, change of medication or other unaccounted systemic conditions and not necessarily related to the periodontal therapy (Table 3. 4).

Table 3. 3 Effect of periodontal therapy on clinical parameters in the immediate group. Values are represented as median (max, min) significant differences between baseline and 6 months after therapy are calculated using Wilcoxon test (significance <0.05). N=20 patients. Abbreviations: PPD, probing pocket depth; CAL, Clinical Attachment level; BOP, bleeding on probing; DAS 28, disease activity score 28; ESR, Erythrocyte sedimentation rate.

	<b>Baseline median (IQR)</b>	<b>6 months median (25, 75 percentile)</b>	<b>Sig two tailed p value</b>
<b>PPD</b>	3.15 (0.57)	2.84 (0.9)	0.01*
<b>CAL</b>	3.42 (0.82)	3.55 (1.8)	0.162
<b>BOP</b>	0.24 (0.26)	0.13 (0.19)	0.0001*
<b>Das 28</b>	5.27 (1.9)	4.06 (1.8)	0.035*
<b>ESR</b>	26.5 (27)	14 (16)	0.062
<b>Tender joints</b>	13.5 (12.3)	6 (10)	0.045*
<b>Swollen joints</b>	1.5 (4)	1 (2)	0.176
<b>CRP</b>	3.9 (14.1)	2.7 (9.8)	0.98

Table 3. 4 Change of clinical parameters in the delayed group. Values are represented as median (max, min) and significant differences between visit 1, baseline (V1) and visit 2, 6 months after therapy (V2) are calculated using Wilcoxon test (significance <0.05). N=22 patients. Abbreviations: PPD, probing pocket depth; CAL, Clinical Attachment level; BOP, bleeding on probing; DAS 28, disease activity score 28; ESR, Erythrocyte sedimentation rate

	<b>V1 median (IQR)</b>	<b>V2 median (IQR)</b>	<b>Sig two tailed p value</b>
<b>PPD</b>	3.15 (2.6, 5.9)	3.4 (0.8)	0.004 *
<b>CAL</b>	3.42 (2.6, 5.9)	3.7 (1)	0.013*
<b>BOP</b>	0.24 (0, 0.6)	0.23 (0.4)	0.14
<b>Das 28</b>	5.27 (3, 7.1)	5.1 (2.9)	0.79
<b>ESR</b>	26.5 (7, 87)	17 (32)	0.011*
<b>Tender joints</b>	13.5 (0, 25)	12 (16)	0.9
<b>Swollen joints</b>	1.5 (0, 12)	0.5 (2)	0.39
<b>CRP</b>	3.9 (0.4, 38.6)	6.09 (0.16, 22.6)	0.98

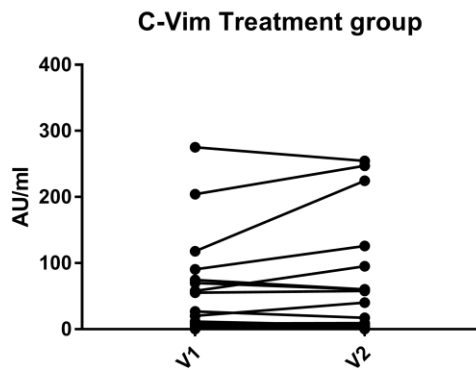


### 3.3.2. Effect of periodontal therapy in levels of antibody to citrullinated proteins

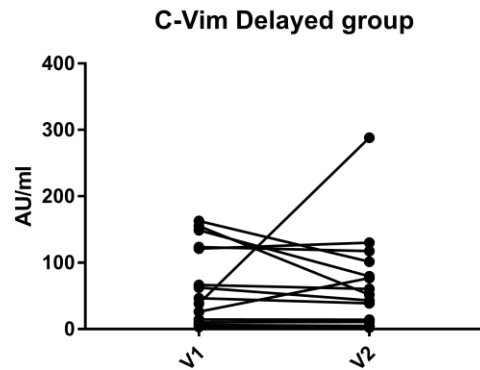
A total of 80 serum samples from 40 RAPD patients collected in two different visits were analysed for quantification of antibodies against human citrullinated proteins. Serum was collected at baseline, on the screening visit (V1) and 6 months later (V2). For the longitudinal study, 20 of these patients were randomised to receive periodontal therapy and 22 patients only received hygiene instructions.

In the treatment group, no clear effect of periodontal therapy on the antibody levels against citrullinated proteins was observed compared to the delayed group (Figure 3. 9-3.9). Although no significant difference was observed between V1 and V2 antibody levels for any of the antigens, some trends could be observed.

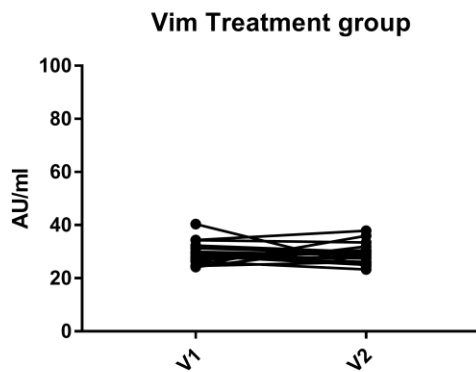
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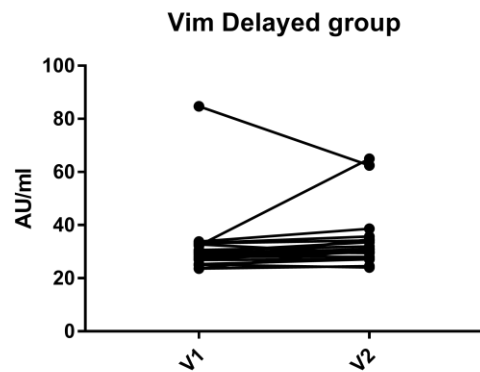
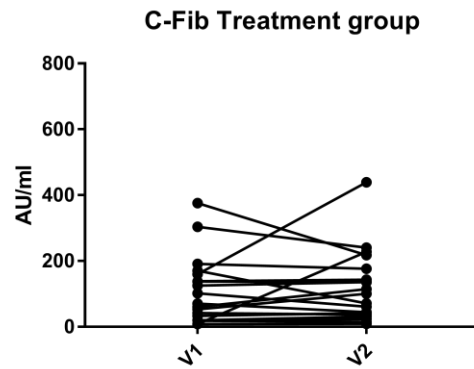
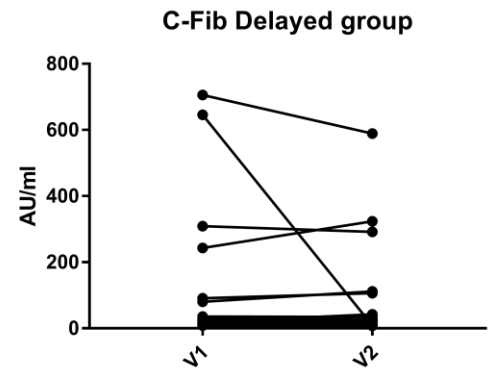


Figure 3. 9 Longitudinal analysis of levels of antibodies to citrullinated and arginated Vimentin. n Treatment group=20; n Delayed group=22. A and b show the antibodies to citrullinated vimentin (C-Vim) in the two randomization group; c and d show the antibodies to the arginated form. V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml) or, if no standards available, Optical Density units (ODs). Statistical test used was Wilcoxon matched pair test (significance  $p < 0.05$ ). Methods described in section 2.2.

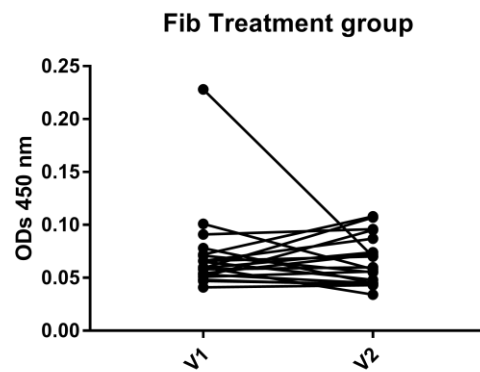
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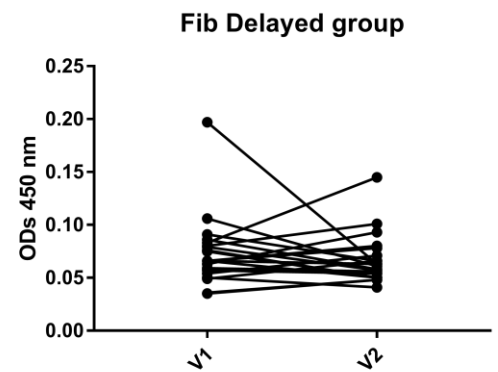
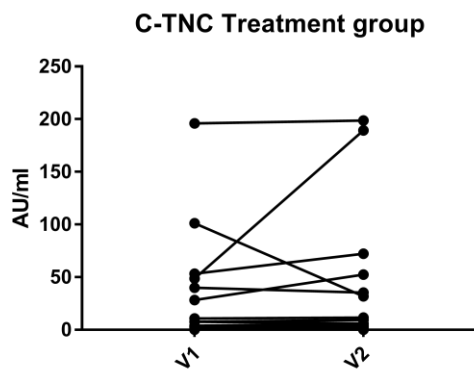
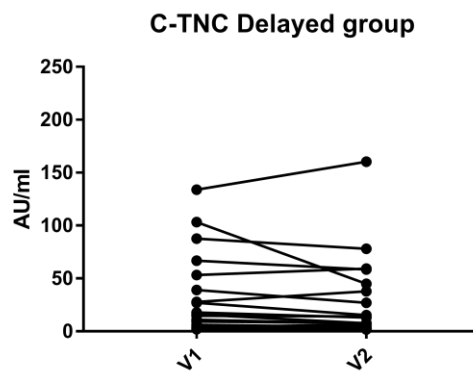


Figure 3. 10 Longitudinal analysis of levels of antibodies to citrullinated and arginated fibrinogen. n Treatment group=20; n Delayed group=22. A and b show the antibodies to citrullinated fibrinogen (C-Fib) in the two randomization group; c and d show the antibodies to the arginated form. V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml) or, if no standards available, Optical Density units (ODs). Statistical test used was Wilcoxon matched pair test (significance  $p < 0.05$ ). Methods described in section 2.2.

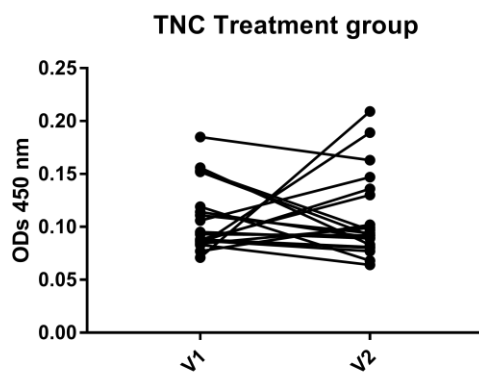
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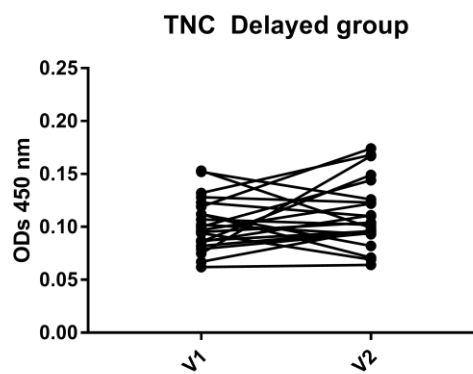


Figure 3. 11 Longitudinal analysis of levels of antibodies to human citrullinated and arginated tenascin. n Treatment group=20; n Delayed group=22. A and b show the antibodies to citrullinated tenascin (C-TNC5) in the two randomization group; c and d show the antibodies to the arginated form. V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml) or, if no standards available, Optical Density units (ODs). Statistical test used was Wilcoxon matched pair test (significance  $p < 0.05$ ). Methods described in section 2.2.

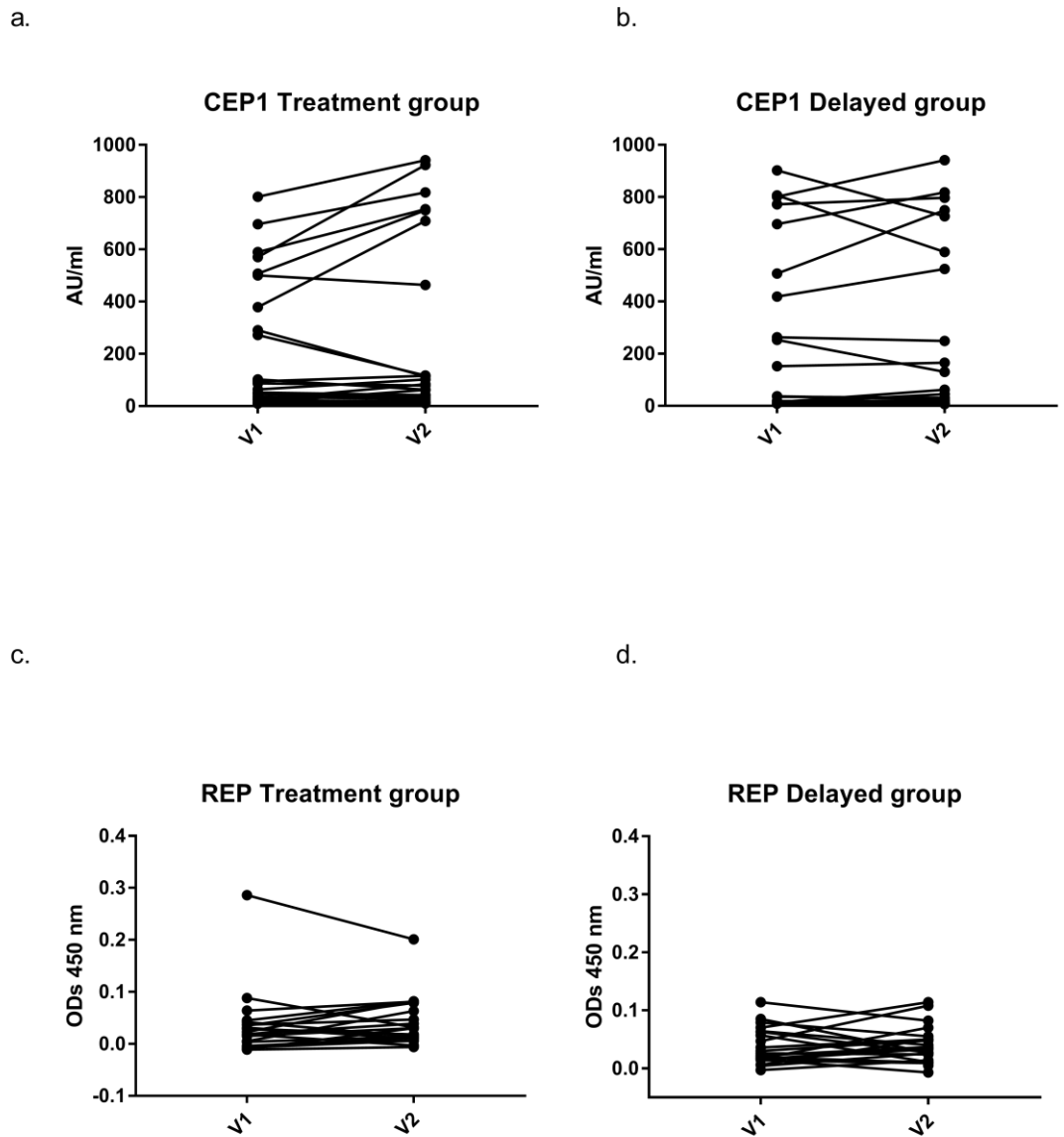
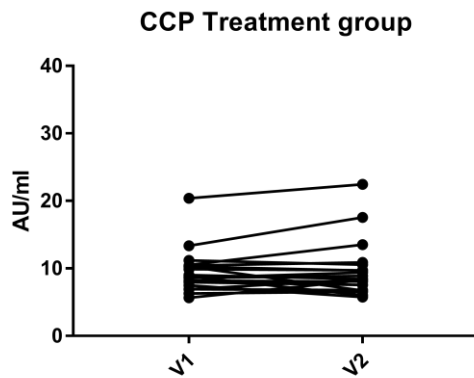


Figure 3. 12 Longitudinal analysis of levels of antibodies to human citrullinated and arginated enolase 1. n Treatment group=20; n Delayed group=22. A and b show the antibodies to citrullinated enolase (CEP) in the two randomization group; c and d show the antibodies to the arginated form. V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml) or, if no standards available, Optical Density units (ODs). Statistical test used was Wilcoxon matched pair test (significance  $p < 0.05$ ). Methods described in section 2.2.

a.



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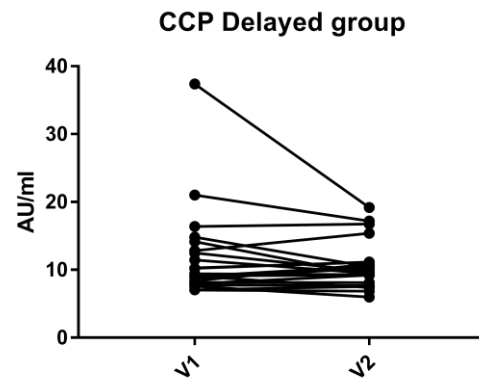
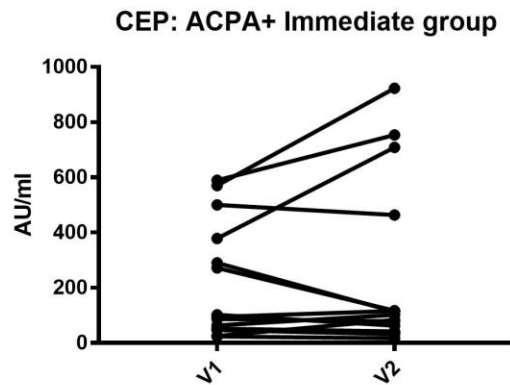


Figure 3. 13 Longitudinal analysis of levels of antibodies to Cyclic Citrullinated Peptide (CCP). n Treatment group=20; n Delayed group=22. A and b show the antibodies to CCP in the two randomization V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml. Statistical test used was Wilcoxon matched pair test (significance  $p < 0.05$ ). Methods described in section 2.2.

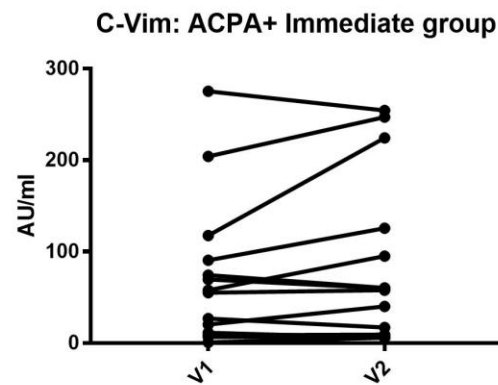
### 3.3.3. Subgroup analysis ACPA+/-

ACPA status from the rheumatologic clinics was available from the patients that were eligible for the longitudinal study. A comparison between the ACPA status taken from different hospitals previous to the enrolment in the study versus the anti-CCP test done in this study, revealed that the two tests gave different classifications in only 6 out of 42 of the patients. Being confident that the two results give the same results in more than 85% of the patients, a subgroup analysis stratified in ACPA status was performed to investigate the effect of periodontal therapy in the two groups. 32 patients were classified as ACPA+, 17 from the delayed group and 15 from the immediate group. Although previous reported that ACPA+ patients that undergo periodontal treatment have a better immunological response, however, this was not reflected in our study (Figure 3. 14).

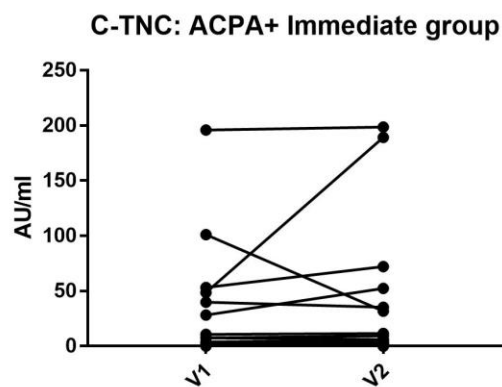
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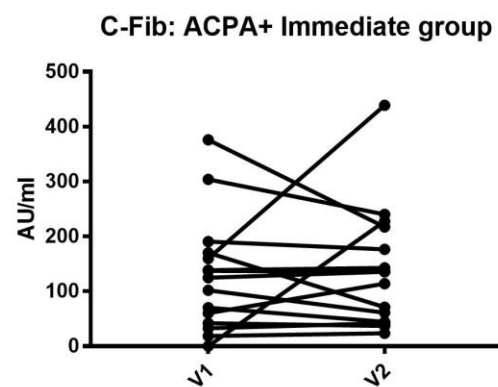


Figure 3. 14 Antibody changes after periodontal treatment in ACPA positive patients. n=15. V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml). Statistical test used was Wilcoxon matched pair test (significance  $p < 0.05$ ). Methods described in section 2.2.

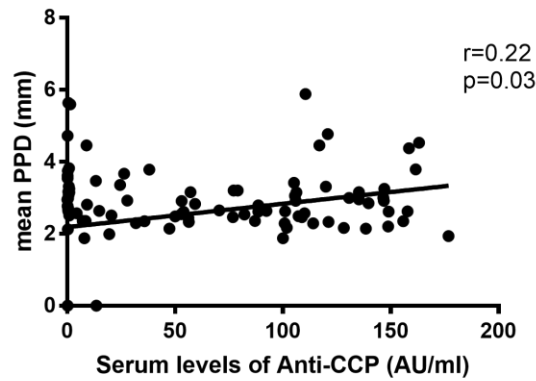


### 3.4. Correlation analysis

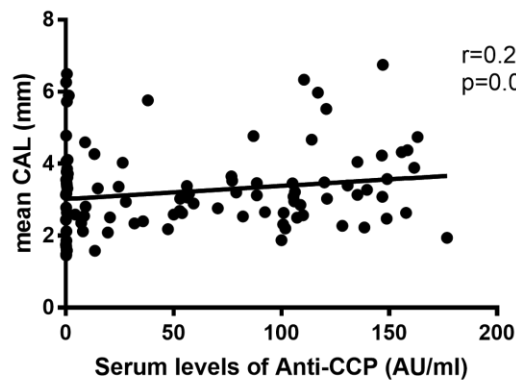
The Spearman rank correlation analysis was performed to investigate the relationship between the ACPA results and the clinical (rheumatologic and periodontal) parameters. No significant ( $p < 0.05$ ) and strong ( $p > 0.7$ ) correlations were found between ACPA production and clinical parameters, but numerous weak significant correlations were found between the different ACPAs tested (anti CCP, CVim, CEP and CFib) and between anti-CCP and the periodontal (PPD and CAL) in all study population and between anti-CCP and rheumatological (ESR) parameters in all RA patients (Figure 3. 15)

Additionally, correlation analysis of the changes in the antibodies after treatment was investigated, testing Spearman's correlation of the difference between clinical data and antibody levels at baseline and 6 months after periodontal treatment. While some significant correlations were found ( $p < 0.05$ ), none of these correlations were strong ( $p > 0.7$ ) (Figure 3. 16)

**a** Correlation analysis between CCP and PPD



**b** Correlation analysis between CCP and CAL



**c** Correlation analysis between CCP and ESR

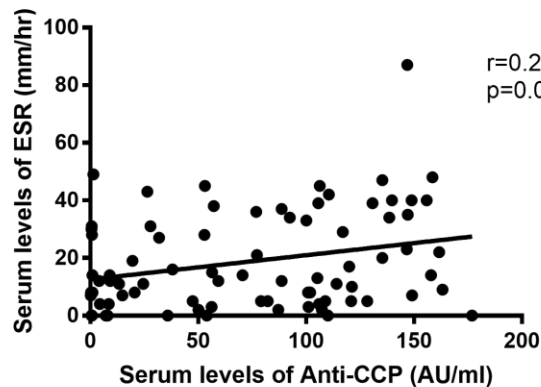
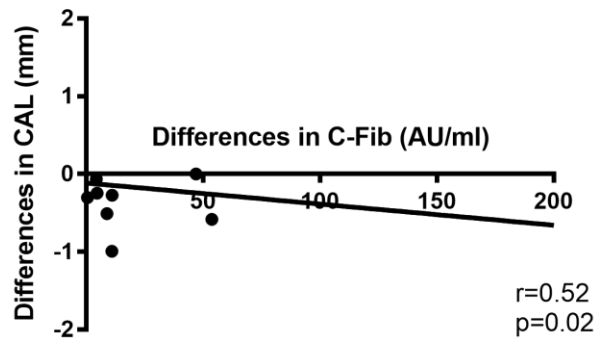


Figure 3. 15 Correlation analysis between serum CCP levels and clinical parameters in study population at baseline ( $n=123$ ). Positive weak but significant ( $p<0.05$ ) correlations were found between levels of CCP and PPD (a), CAL (b) and ESR ( $n=80$ ) (c). Spearman's correlation was tested.

**a Correlation analysis between changes in CAL and C-Fib**



**b**

**Correlation analysis between changes in BoP and C-Vim**

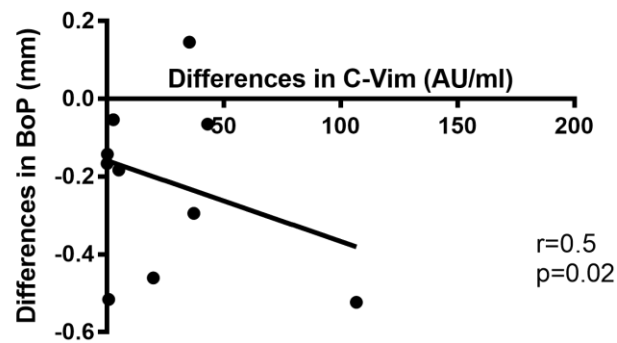


Figure 3. 16 Correlation analysis of the changes before and after periodontal therapy in serum ACPAs levels and clinical parameters (n=20). Negative weak but significant ( $P<0.05$ ) correlations were found between changes in CAL and C-Fib levels (a) and between C-Vim levles and BoP (b). Spearman's correlation was tested.

### 3.5. Discussion

The hypothesis of this study was that, in rheumatoid arthritis, patients with periodontitis have a higher level of antibodies to citrullinated proteins compared to those with healthy periodontium and that, after periodontal therapy, these antibody levels are reduced. Our results did not support this hypothesis.

#### 3.5.1. Cross-sectional study results

While other studies have investigated the ACPA reaction in RA through antibody levels to anti-CCP only, it is important to test individual ACPA fine specificities together with anti-CCP and peptide controls in RA (Montgomery, Venables et al. 2013). To our knowledge, the study presented here analyses the widest range of antibodies to human and *P. gingivalis* -related peptides, in different patients groups with and without RA and periodontitis. Also it is the only study that investigates the effect of periodontal treatment in antibodies to this range of specific antigens.

Severity of periodontitis did not seem to be associated with anti-CCP positive (anti-CCP antibodies > 98<sup>th</sup> percentile of health) RA as previously described (Dissick, Redman et al. 2010) as the percentage of moderate and severe periodontitis in the anti-CCP negative group was greater than that in the anti-CCP positive group (31% compared to 18%). These findings contradict the results from the pilot study of Dissik and collaborators. However, the number of anti-CCP

negative patients in the RA group was very low and the analysis used here did not correct for different group sizes, so the results of these comparisons may not be as reliable as the ones from larger studies. Also, in this chapter severity of periodontitis was defined using the described modified definition of Eke&Page (Chapter 2). Yet, other grouping factors could be use such as number or percentage of sites with PPD > 5mm, which could show different results.

Our results show that the antibody levels in the RAPD group were similar compared to the periodontally healthy RA group for the antigens tested in the study. This is also supported by the lack of difference in antibody response between periodontally healthy patients and the moderate to severe periodontitis groups. Whilst there are more patients ACPA-positive in the RAPD group compared to the RA noPD group with antibodies against C-Fib and CTNC5, the results for anti-CEP and C-Vim show the opposite. This slight difference between the analytes could be explained through the differences in the association to the human leukocyte antigen-shared epitope (HLA-SE). The development of anti-CEP and C-Vim antibodies are strongly associated with HLA-SE (Lundberg, Bengtsson et al. 2012),(Kokkonen, Brink et al. 2015), but not C-Fib and there is no evidence of the association with C-TNC5. When no genetic factors are associated with the presence of antibodies, the environmental factor would become a fundamental feature for the production of antibodies, which in this case would be the presence of periodontitis. This could explain why, in RA, a higher proportion of patients with antibodies against C-TNC5 and C-Fib in the periodontitis group is found but not with anti-CEP and C-Vim antibodies.

Although cross-reactivity of the different ACPAs specificities is being widely debated, a previous study demonstrated only limited cross-reactivity between the specificities investigated here (low cross-reactivity degree between C-Fib and CEP only) (Lundberg, Bengtsson et al. 2012). In our study, only 7 patients from all anti-CCP positive patients (n=68) expressed antibodies to all the other antigens indicating low overlapping. Also some of these anti-CCP positive patients were not positive for any of the citrullinated antigens studied (n=6), showing that there are still some citrullinated antigens that need to be investigated. There did not seem to be a clear relationship with periodontal status as patients with and without periodontitis were equally distributed.

Although the number of anti-CCP negative RA patients in our study is low (n=18), 4 of the anti-CCP negative patients expressed antibodies against at least one of the other citrullinated peptides (one patient anti-CCP negative was positive for anti-C-Fib, C-Vim and C-TNC5), indicating that anti-CCP may fail to pick antibodies to citrullinated peptides and specific antigens should be investigated.

In systemically healthy patients no association was found between ACPA production and chronic periodontitis, demonstrating similar findings to Hendler and collaborators (Hendler, Mulli et al. 2010). However this contradicts the results shown in the study from De Pablo and collaborators, which found a significant increase of antibody production to citrullinated and uncitrullinated antigens in periodontitis patients compared to controls (de Pablo, Dietrich et al. 2014). Also

Lappin and collaborators found significantly higher levels of CEP and anti-CCP in patients with periodontitis compared to controls (Lappin, Apatzidou et al. 2013). This difference might be due differences to the number of patients studied as, in the de Pablo study there were almost 200 patients enrolled, compared to less than 40 patients in the two studies that did not find the association.

The lack of strong and significant correlations between antibody levels and clinical parameters contradicts previous studies (Fisher, Plant et al. 2011, Potikuri, Dannana et al. 2012, Lee, Choi et al. 2015). In the study conducted by Lee and collaborators, results show an association between antibodies to enolase and periodontal parameters. However, the correlations found in this paper and those found by Potikuri and colleagues, are also weak, with  $r$  values ranging between 0.05 and 0.18.

Although the level of antibodies to uncitrullinated proteins are lower than the levels of antibodies to the citrullinated forms, our study also showed higher levels of anti-uncitrullinated peptides in the RA groups compared to the systemically healthy controls. Following the theory described by de Pablo and collaborators (de Pablo, Dietrich et al. 2014), this might be due to a loss of tolerance to uncitrullinated proteins in RA due to periodontitis. However, as the amount of peptide available for this experiment was very limited, it was not possible to prove that the lack of antibody positivity to the uncitrullinated peptides was not due to a methodology problem such as poor peptide binding so this remains a possible explanation for the results found.

### 3.5.2. Longitudinal study results

Our hypothesis, following the theory of extra-articular citrullination in periodontitis triggering and exacerbating RA, was that periodontal treatment could bring down levels of citrullination and therefore, the production of ACPAs. This is supported by previous research that found that supragingival cleaning decreased levels of citrulline in RA patients (Okada, Kobayashi et al. 2013). However, our results did not support our hypothesis, as no changes were found in the ACPA production after periodontal therapy in any of the peptides tested.

Another recent study published by Shimada and collaborators failed to find a reduction in anti-CCP levels and anti PPAD in RA 2 months after periodontal therapy (Shimada, Kobayashi et al. 2016). These negative results contradict those from Okada et al (Okada, Kobayashi et al. 2013), who found a significant decrease in antibodies to *P. gingivalis* in RA in the treatment group compared to control. These opposing results could be explained through the different methodology, as the study from Okada used a mild definition of periodontitis and provided supragingival scaling, while the paper from Shamada and our paper includes moderate to severe periodontitis patients and provided subgingival periodontal therapy. While it seems that in mild cases of periodontitis a simple supragingival scaling might be enough to reduce the level of *P. gingivalis* and therefore reduce citrullination, in periodontitis it might be more complex to demonstrate this effect.



### 3.5.3. Limitations of the study

Although our hypothesis was contradicted by the results, there are limitations in our study that could explain such negative results. Due to dropouts and difficulties in sample collection, only 20 patients from each treatment arm were included for the longitudinal study, while the study from Shimada (also showing negative results) includes 24 patients. This small sample size makes it very challenging to find any statistical significance when comparing the groups.

Also, during the duration of the study, many of the subjects suffered changes in the medications or flair ups of RA. These fluctuations might be causing the abrupt changes observed in the antibody levels of some patients and diminishing the effect of periodontal therapy. However, these variations are very difficult to control in this type of study and due to the small sample size, doing regression or subgrouping was not appropriate.

Moreover, although we see a significant reduction of the periodontal parameters after periodontal therapy, there are only 9 patients that achieve mild periodontitis or health, while the majority (65%), even with a significant reduction of bleeding and pocket depth, would still be classified as periodontitis patients. This might reflect the need of a more aggressive treatment and adjunctive treatment in the follow up visits, to ensure that all patients that fell in the treatment group finished the study with periodontal health.

Furthermore, 6 months might not be enough time to observe an the antibody response even when the stimulus has been reduced. At the beginning of an immune response, plasma cells only live for a few day or weeks. As B cells mature into plasma cells, they can survive for months or years, prolonging the secretion of antibodies (Bernasconi, Traggiai et al. 2002). For this reason, a longer and larger study, with patients progressing from moderate-severe periodontitis to health, might be needed to observe a possible change in the antibody response after periodontal treatment

On the other hand, even considering the limitations of our study, periodontal therapy might not have an effect on antibody levels in RA as it might produce an effect too small to show a change in the B cell behaviour and antibody production. While in our study, there was a significant reduction of DAS28 after periodontal therapy, the mean change dropped from 4.6 to 4.06, clinically this is a very small difference. Although the benefits of maintaining oral health are clear, the effect of periodontal therapy in RA parameters have not yet been elucidated and it might be that considering the fluctuating and exacerbated auto-immune response observed in RA, once the autoimmune response has been settled over years of exposure to the triggering factors, eliminating periodontitis might not be enough for the immune system to reverse to a healthy status. As memory B cells persist years or even for a lifetime, once they have been activated involving a particular antigen (which could be citrullinated proteins by *P. gingivalis*) in a primary response, the continuous citrullination found in rheumatic joints will activate B cells that respond quickly upon second exposures to the antigen. Following this

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hypothesis, removing *Porphyromonas gingivalis* with periodontal therapy, although beneficial for the oral health and decreasing the inflammation, might not change the antibody response in RA.

# **4. RESULTS - CIRCULATING ANTIBODIES TO PERIODONTAL BACTERIA IN RA AND PERIODONTITIS**

#### 4.1. Introduction

The hypothesis of the study presented in this chapter was that in rheumatoid arthritis there is an elevated production of serum antibodies to periodontal bacteria compared to controls and that, in RA patients with periodontitis undergoing periodontal therapy, the level of these antibodies could be reduced. This is important, as periodontal bacteria have been described to be a key driver of the autoimmune response that originates and perpetuates the state of chronic inflammation in RA (Scher, Bretz et al. 2014).

The aims were:

- 1) To compare the level of serum antibodies to outer-membrane antigens (OMAs) from periodontal bacteria and *P. gingivalis* -related antigens in groups of patients diagnosed with RA and the appropriate control groups with and without periodontitis.
- 2) To analyse the effect of periodontal therapy on the level of these antibodies in RA patients with periodontitis compared to a control group that did not receive such therapy.

This chapter presents the results from serum antibody ELISAs to OMAs from *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* conducted at the University of Nebraska Medical Center (methods described in

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section 2.5) through a collaboration with Dr Ted Mikuls, and the results from serum antibody ELISAs to four *P. gingivalis* related proteins: Peptidyl Arginine Deiminase (PPAD) and its immunodominant citrullinated (CPP3) and arginated (RPP3) epitopes, gingipain (RgpB) and *P. gingivalis* enolase (ELISA methods are described in detail in chapter 2.2). *P. gingivalis* -antigens were provided by Prof Patrick Venables (Oxford University).

## **4.2. Antibodies to PG-related antigens**

### **4.2.1. Patient background and clinical results**

For this project, sera collected from 124 patients from the OPERA and INSPIRED cohorts (recruitment and study population explained in chapter 2) were analysed, of which 87 patients were diagnosed with RA and 37 patients were systemically healthy. Clinical data from the different patient groups are described in section 3.2 (Table 3.1).

### **4.2.2. Cross-sectional group comparison**

In the RAPD group, antibody levels against all the PG-related peptides were higher than in the RA noPD group, although these differences were not statistically significant (Figure 4. 1). Interestingly, the RA group with healthy periodontium also showed higher levels of antibodies against PPAD close to significance ( $p=0.06$ ), RPP3 and CPP3, compared to systemically healthy patients with PD. This suggests that, in RA, PPAD and its epitopes, could be

recognized as an antigen in a similar way to human ACPAs even with healthy periodontium.

Previously, de Pablo et. al represented similar data as the percentage of patients that have ACPA levels over the 98th percentile of healthy controls (antibody positivity). The same analysis was applied here resulting in non-significant higher percentage of patients in the RA (compared to systemically healthy) groups, for the antibody response against PPAD, CPP3, RPP3 and PG Enolase, even in the periodontally healthy RA group (Figure 4. 2)

Although the antibody reaction to most individual PG-antigens such as PPAD, CPP3 and RPP3 was non-significantly higher in both RA groups, with or without periodontitis, this was not observed in the case of antibodies to RgpB, which were elevated in the periodontitis groups, both RA and systemically healthy, compared to the periodontally healthy groups. This shows that in RA, PG antigens related to PPAD act as antigen but not RgpB, which only acts as antigen in periodontitis patients.

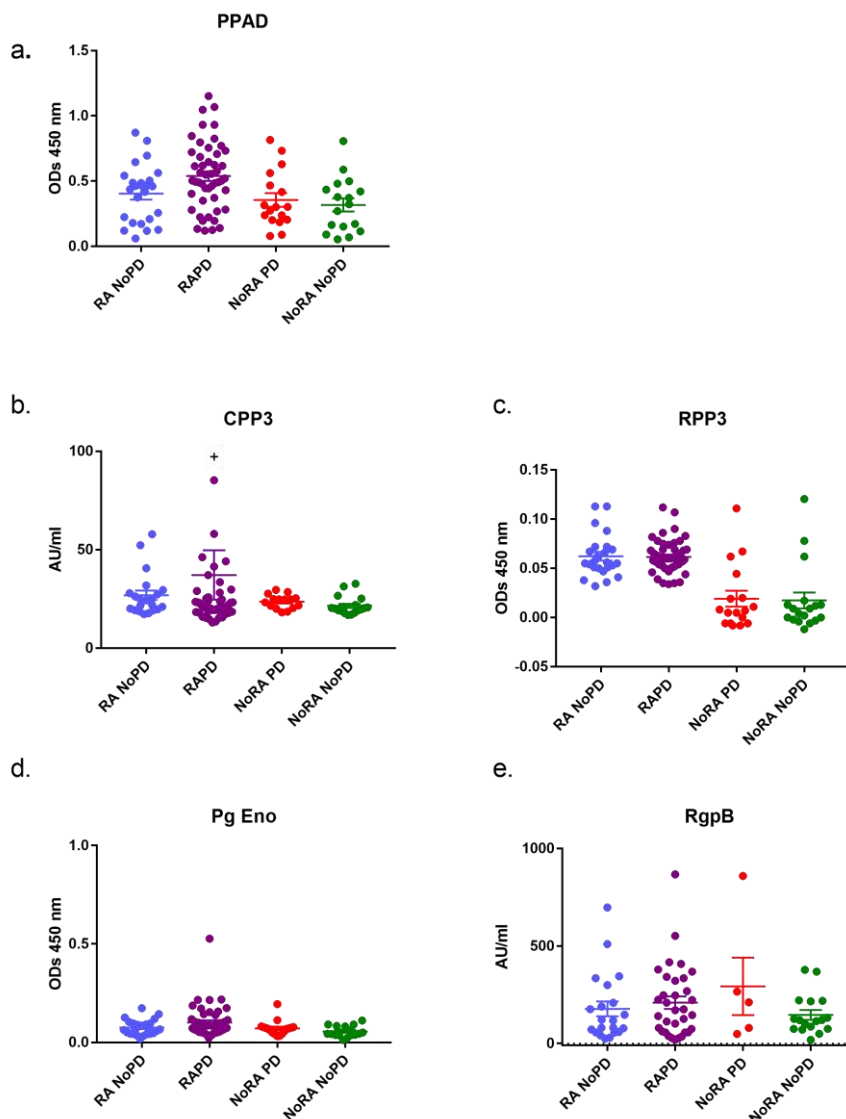


Figure 4. 1 Level of antibodies in the different study groups: Rheumatoid arthritis with healthy periodontium (RA noPD) (n=24); baseline data from RA with Periodontitis (RAPD) (n=50); systemically healthy with periodontitis (NoRA PD) (n=17); systemically and periodontally healthy controls (NoRA NoPD) (n=18). ). Data represented in scatter plot graphs and colored lines show the group mean and SEM. Each individual graph represents the results for each peptide: a. *Porphyromonas gingivalis* peptidylarginine deiminase (PPAD); b. citrullinated and c. arginated epitope from PPAD (CPP3/RPP3); d. enolase from PG; e. *P. gingivalis* virulence factor arginine gingipainB (RgpB). Significant differences between groups were analysed using the Kruskal-Wallis test and Tukey's comparison (singinificant if  $p < 0.05$ , + symbol represents an outlier). Methods described in section 2.2.



### Percentage of patients with Pg-antibody levels over 98 healthy percentile

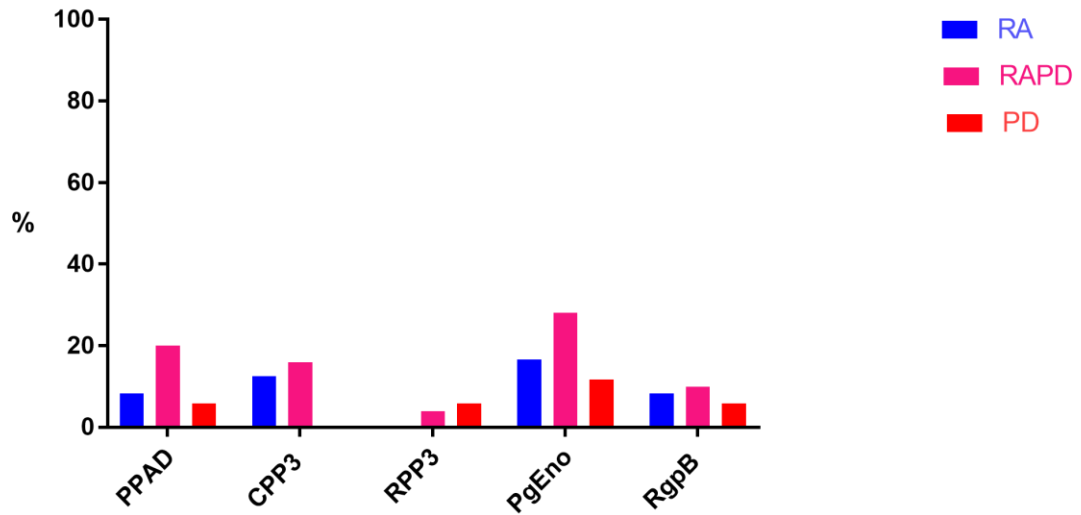


Figure 4. 2 Percentage of patients with antibody levels above the 98th percentile of healthy controls. Antibody levels tested against: Whole Peptidyl-arginine deiminase (PPAD), CPP3 (citrullinated peptide3 from PPAD), RPP3 (arginated peptide 3 from PPAD), PG-enolase and arginine-gingipain B (RgpB). Rheumatoid arthritis with healthy periodontium (RA noPD) (n=24); baseline data from RA with Periodontitis (RAPD) (n=50); systemically healthy with periodontitis (NoRA PD) (n=17); systemically and periodontally healthy controls (NoRA NoPD) (n=18). Methods described in section 2.2. Significance defined as  $p < 0.05$ , test used Pearsons Chi-squared test. Abbreviations: ACPA, anti-citrullinated protein antibody; RA, rheumatoid arthritis; PD, periodontitis

#### 4.2.3. Subgroup analysis

##### 4.2.3.1. RA, periodontitis and smoking analysis

The results from the analysis based on systemic disease (RA vs noRA) showed that RA patients had significantly higher levels of antibodies to PPAD ( $p=0.0013$ ), PG-enolase ( $p=0.0031$ ) and RPP3 ( $p=0.001$ ). While a trend towards an increased antibody reaction against anti-PG antigens is seen with increasing severity of periodontal disease (Figure 4. 3), only anti-PG enolase and PPAD antibodies were significantly increased in the moderate/severe periodontitis patients compared to controls (PG Eno  $p=0.002$ ; PPAD  $p=0.001$ ). However, no significant differences were found in the antibody levels to PG-antigens between smokers, former smokers or patients that never smoked. This was an interesting finding as it was previously shown that in pre-RA patients, anti-RgpB and anti-PPAD antibodies were higher in smokers (Fisher, Cartwright et al. 2015) although smoking has been shown to reduce concentration of IgG antibodies in general due to its depletive effect on the immune system (Barbour, Nakashima et al. 1997) (Kinane and Chestnutt 2000).

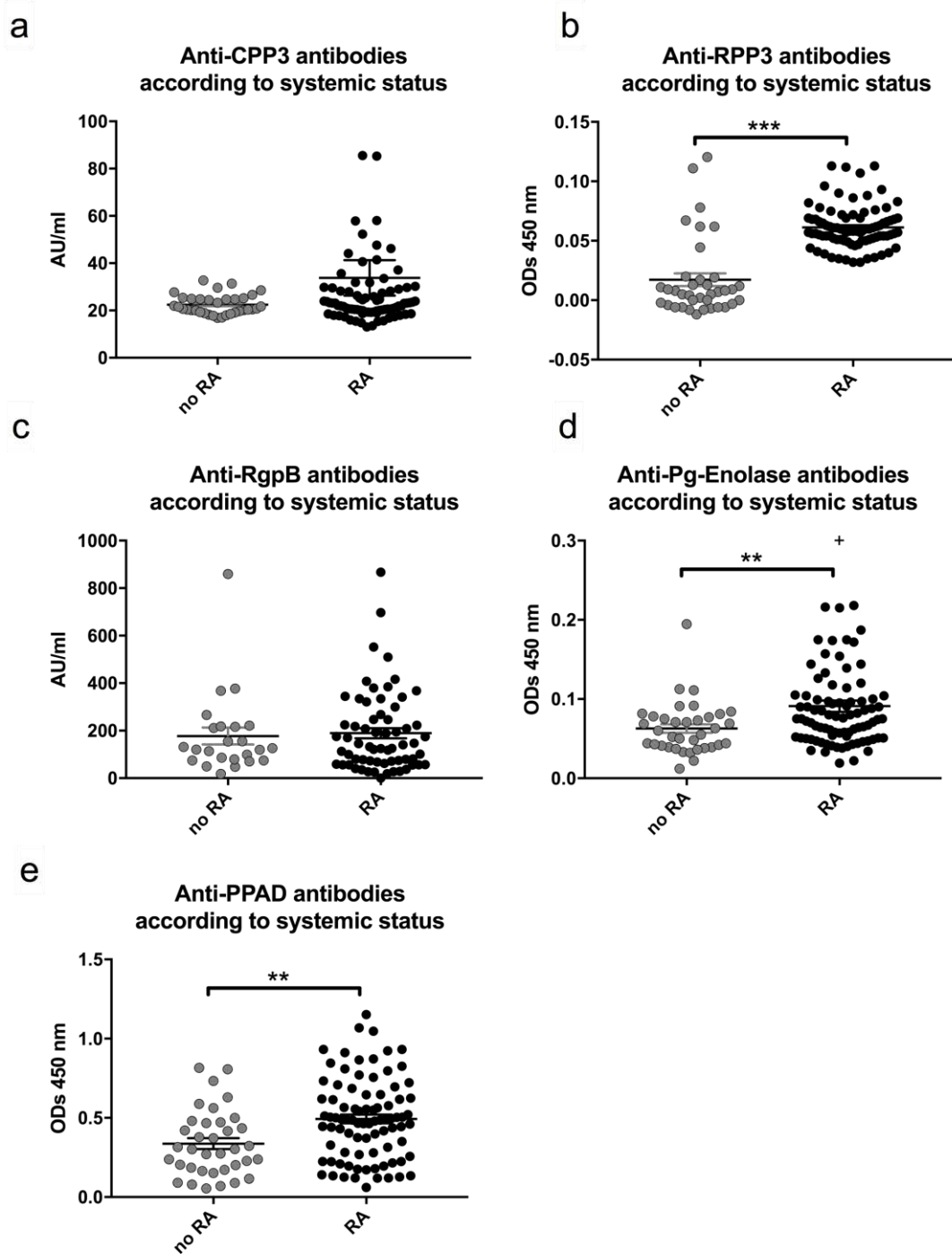


Figure 4. 3 Antibodies to PG antigens in RA patients compared to controls. PG-antigens investigated: a. citrullinated epitope 3; b. arginated epitope 3; c. gingipain B; d. enolase from PG; e. peptidyl arginine deiminase from PG. n RA= 88, n noRA= 36. Data represented as scatter plots and lines represent the mean and SEM. Significance represented as \* if  $p < 0.05$ , \*\* if  $p < 0.01$ . Statistical test used: Mann-Whitney test. ELISA methods described in section 2.2. Abbreviations: PG, *Porphyromonas gingivalis*.

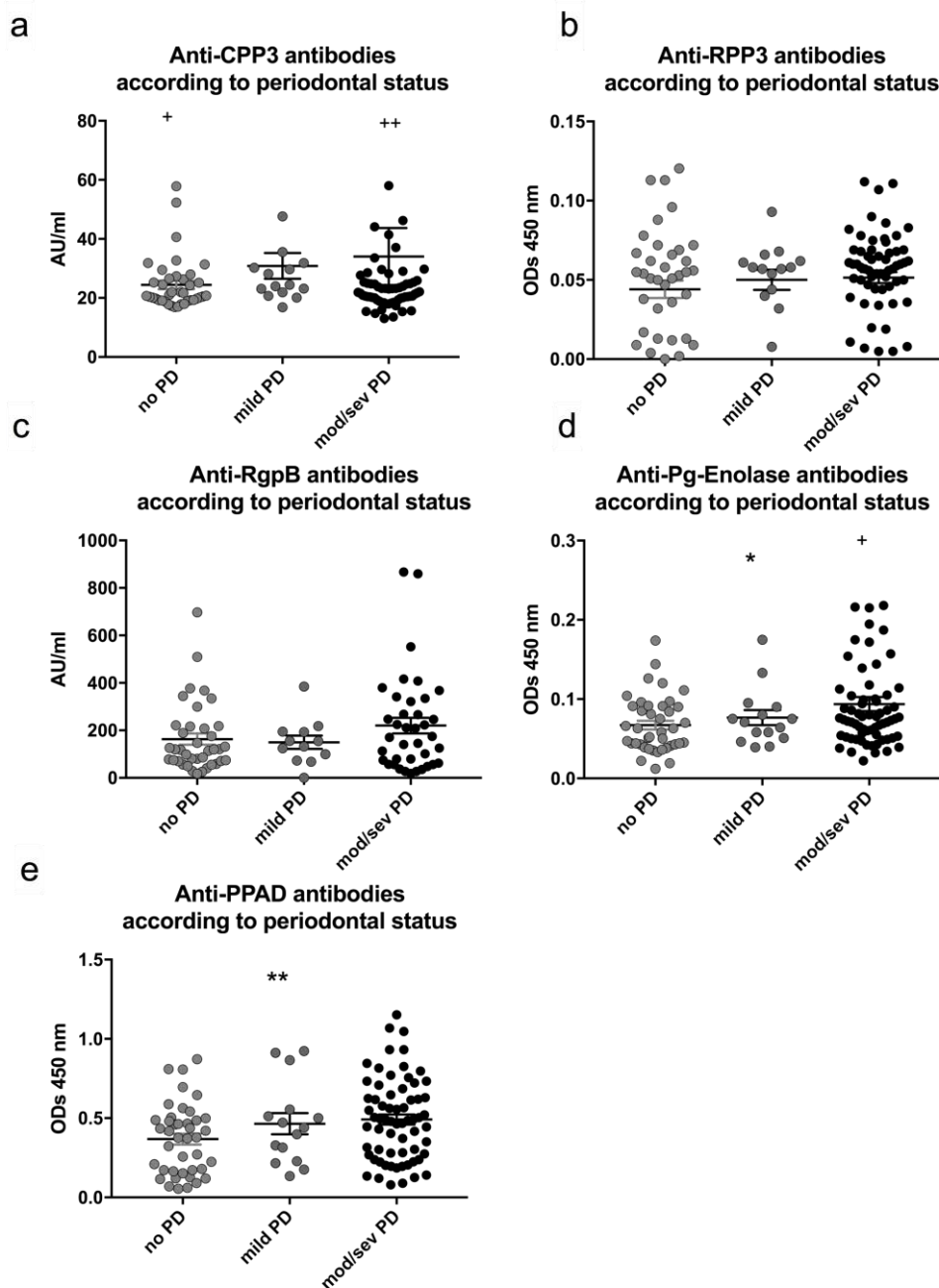


Figure 4. 4 Antibodies to PG antigens according to periodontal status: no periodontitis (n noPD=42), mild periodontitis (n mild PD= 15), moderate and severe periodontitis (n mod/sev PD=67) PG-antigens investigated: a. citrullinated epitope 3; b. arginated epitope 3; c. gingipain B; d. enolase from PG; e. peptidyl arginine deimanase from PG. Data represented as scatter plots and lines represent the mean and SEM. Significance represented as \* if  $p < 0.05$ , \*\* if  $p < 0.01$ . Statistical test used: Kruskal Wallis with Dunn's multiple comparison. ELISA methods described in section 2.2. Abbreviations: PD, periodontitis; PG, *Porphyromonas gingivalis*.

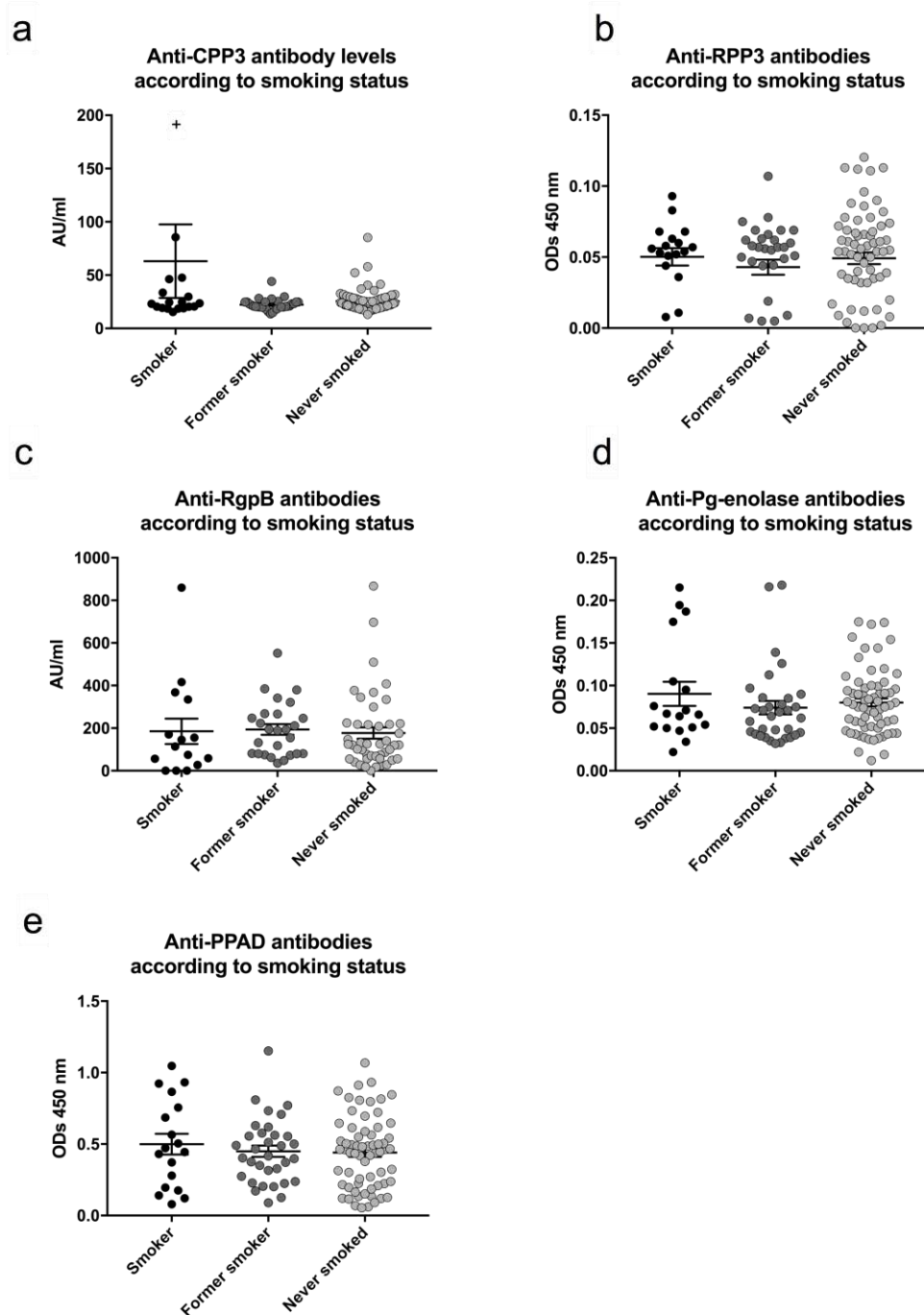


Figure 4. 5 Antibodies to PG antigens according to smoking status: smokers (n=18), former smokers (n PD=33), never smoked (n =67) PG-antigens investigated: a. citrullinated epitope 3; b. arginated epitope 3; c. gingipain B; d. enolase from PG; e. peptidyl arginine deiminase from PG. Data represented as scatter plots and lines represent the mean and SEM. Significance represented as \* if  $p < 0.05$ , \*\* if  $p < 0.01$ . Statistical test used: Kruskal Wallis with Dunn's multiple comparison. ELISA methods described in section 2.2. Abbreviations: PG, *Porphyromonas gingivalis*

#### 4.2.3.2. ACPA+ and ACPA- analysis

There is data that suggest that ACPA positive and negative RA patients respond differently to therapy (van Dongen, van Aken et al. 2007) and have distinct pathophysiology and risk factors (Daha and Toes 2011). For that reason, a subgroup analysis stratified by ACPA status was used, showing that anti-CCP negative patients with healthy periodontium did not have antibodies to PPAD, CPP3, RPP3 or PG-Enolase (Figure 4. 6). In contrast, between 10 and 20% of the anti-CCP+ patients with healthy periodontium had antibodies to PPAD, CPP3 and RPP3.

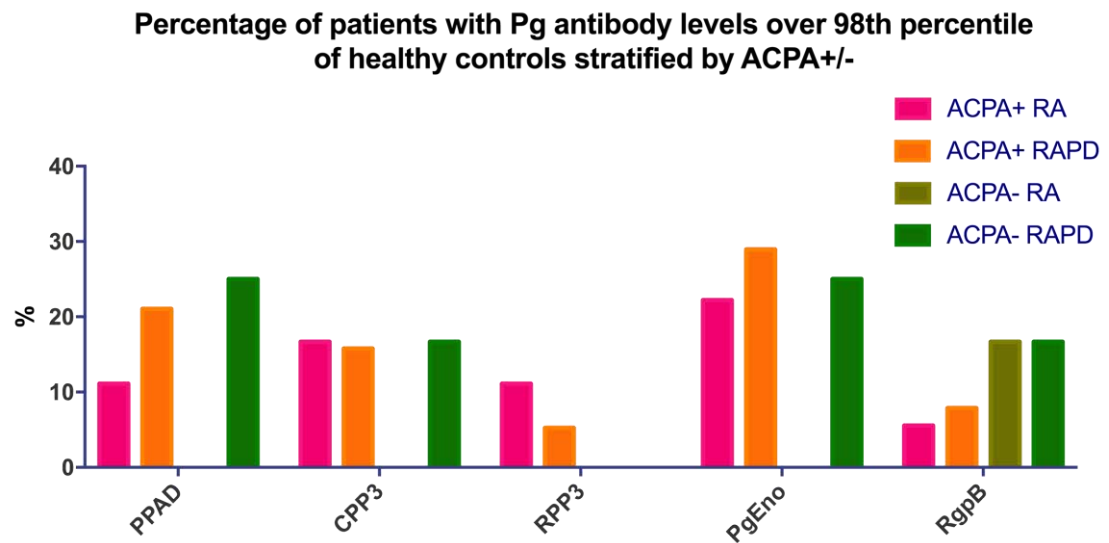


Figure 4. 6 Percentage of patients with antibody levels over the 98<sup>th</sup> percentile of healthy controls, stratified by ACPA positive and negative. Antibody levels tested against: Whole Peptidyl-arginine deiminase (PPAD), CPP3 (citrullinated peptide3 from PPAD), RPP3 (arginated peptide 3 from PPAD), PG-enolase and arginine- gingipain B (RgpB). Data represented in column graph, showing mean group and SEM. Rheumatoid arthritis with healthy periodontium (RA noPD) (n=24); baseline data from RA with Periodontitis (RAPD) (n=50); systemically healthy with periodontitis (NoRA PD) (n=17); systemically and periodontally healthy controls (NoRA NoPD) (n=18). Methods described in section 2.2. No significant differences were found between the RAPD and RA groups in either ACPA positive or negative stratifications. Significance defined as  $p < 0.05$ , test used Pearsons Chi-squared test. Abbreviations: ACPA, anti-citrullinated protein antibody; RA, rheumatoid arthritis; PD, periodontitis

#### **4.3. Antibodies to OMA from *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacteria nucleatum***

Following the methods previously described by Mikuls and collaborators (Mikuls, Payne et al. 2014), antibodies to outer membrane antigens (OMA) from periodontal bacteria were semi-quantified (methods explained in section 1.2). The aim of this experiment to measure antibodies against PG, and antibodies to FN and PI were also analysed as both bacteria are closely associated with PG in the deep pocket biofilm (Holt and Ebersole 2005).

##### **4.3.1. Patient background**

A total of 239 serum samples from 197 patients were analysed in this study from the OPERA and INSPIRED trials (described chapter 2.1, Figure 2.1). From 142 RA patients, 30 were diagnosed with RA and healthy periodontium (RA noPD), 87 were diagnosed with RA and moderate/severe periodontitis (RAPD) and those with mild periodontitis were excluded for the baseline comparison (n=26) but the patients are included in Figure 4.10 for the periodontal status comparison. From a total of 28 patients systemically healthy, 18 were classified as periodontitis patients (NoRA PD) and 19 classified as periodontally healthy controls (NoRA noPD).



This subgroup of OPERA patients was selected from patients whose samples were used for the antibody analysis described in chapter 3 and for the microbiome analysis is described in chapter 5. This enabled the comparison between these results and the rest of the antibody levels and also the correlation between antibodies to membrane proteins to these 3 bacteria in sera and the relative abundance of these bacteria in subgingival plaque found with 16S analysis.

Table 4. 1 Clinical characteristics at baseline of study population groups (n=155). Ordinal data is represented as mean and IQR. Nominal data is represented as percentages.

	RAPD (n=87)	RAnoPD (n=30)	noRAPD (n=18)	NoRA NoPD (n=20)
Female proportion	71.3%	73.3%	55.6%	63%
Ethnicity				
-%Asian	13.8%	4%	5.6%	11%
-%White	40%	90%	78%	89%
-%Black	4.6%	0%	11%	0
Smoker				
-%current	21.8%	10%	5.6%	5%
-%former	32.2%	26.6%	55.6%	11%
-% never	46%	63.3%	33.3%	84%
Alcohol				
-%Never	37%	23%	11%	15%
-%1-4	56%	46%	16%	65%
times/month	3%	20%	44%	15%
-%1-4	4%	10%	16.7%	5%
times/week				
-% >4 times/week				
Weight	74.4	77.35 (30.7)	82.6 (27)	76.42(24)
Age	57 (16)	61.5 (17)	48(8.5)	37(15)
PPD (median, IQR)	3.01 (0.74)	2.26 (0.38)	3.17(1)	1.56(0.3)
BoP	0.24 (0.07)	0.24 (0.09)	0.39(0.25)	0.02(0.11)
CAL	3.29 (1.09)	2.24 (0.8)	3.9(1.9)	1.7(0.28)
DAS28	3.49 (3.8)	3.06 (2.31)	-	-
ESR	11 (29)	8 (24)	-	-
VAS	55 (49)	41 (39)	-	-
Tender joints	7 (15)	4.5 (10)	-	-
Swollen joints	1 (2)	0.1 (2)	-	-

#### 4.3.2. Antibody levels to bacterial proteins group comparison

No significant differences were found in antibody levels across the 4 groups, for anti-OMAs from PG and PI and significantly higher levels of anti-FN were found in RAPD compared to RAnoPD (Figure 4. 7).

Antibodies to PG were elevated in the two periodontitis groups (RAPD and NoRAPD) and the highest levels were found in the NoRAPD with no significant differences to the RAPD group (Figure 4. 7.a). However, no significant differences were found among the study groups.

No significant differences were found in the anti-PI levels among the study groups (Figure 4. 7.b). Although the patients with the highest levels belonged to the RA groups, the two systemically healthy groups had slightly elevated mean antibodies.

Patients with RA and PD had the highest levels of antibodies against FN compared to the rest of the groups and the difference between the RAPD and RAnoPD was significant (Kruskal Wallis with Dunn's test,  $p$  adjusted=0.014). However, anti-FN was not elevated in the PD group systemically healthy compared to periodontally healthy (Figure 4. 7.c).

Combining the two groups of periodontitis patients and the two groups periodontally healthy, significantly higher levels of anti-PG were found to be associated with periodontitis (Mann-Whitney test,  $p=0.005$ ), and low levels were found in the periodontally healthy groups (Figure 4. 8). However, similar levels were found when grouping patients by systemic condition. This represents an antibody reaction to PG associated to periodontitis patients and not related to RA.

Level of antibodies against PI were similar in all the groups, independently of periodontal status and slightly elevated in the RA group compared to systemically healthy (Mann-Whitney test,  $p=0.049$ ). Antibody levels against FN were elevated in the periodontitis group compared to periodontally healthy (Mann-Whitney test,  $p=0.014$ ) but not in the RA group compared to the systemically healthy, suggesting that anti-FN is associated to periodontitis and not to RA.

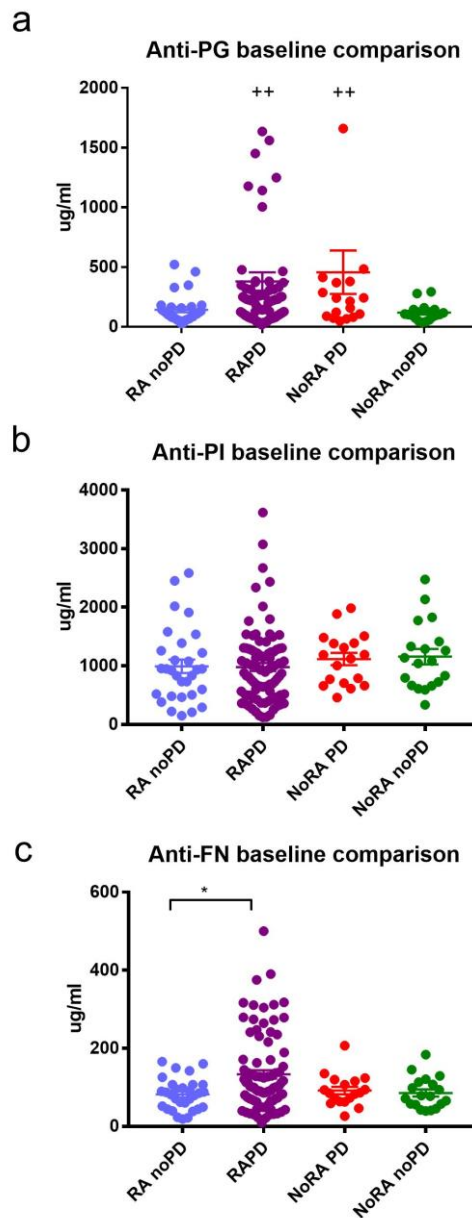


Figure 4. 7 Levels of serum antibodies to outer-membrane antigens (OMA) from from PG, FN and PI in the different study groups. Data represented in ug/ml as scatter plot graphs and colored lines show the group mean and SEM. Each individual graph represents the results for each OMA: a. *Porphyromonas gingivalis* b., *Prevotella intermedia*, c. *Fusobacterium nucleatum*; Significant differences between groups were analysed using the Kruskal-Wallis test and Tukey's comparison (singinficant if  $p < 0.05$ , + symbol represents an outlier). Methods described in section 2.2. Abbreviations: PG, *Porphyromonas gingivalis*; PI, *Prevotella intermedia*; FN, *Fusobacterium nucleatum*; RA, rheumatoid arthritis; PD, periodontitis.

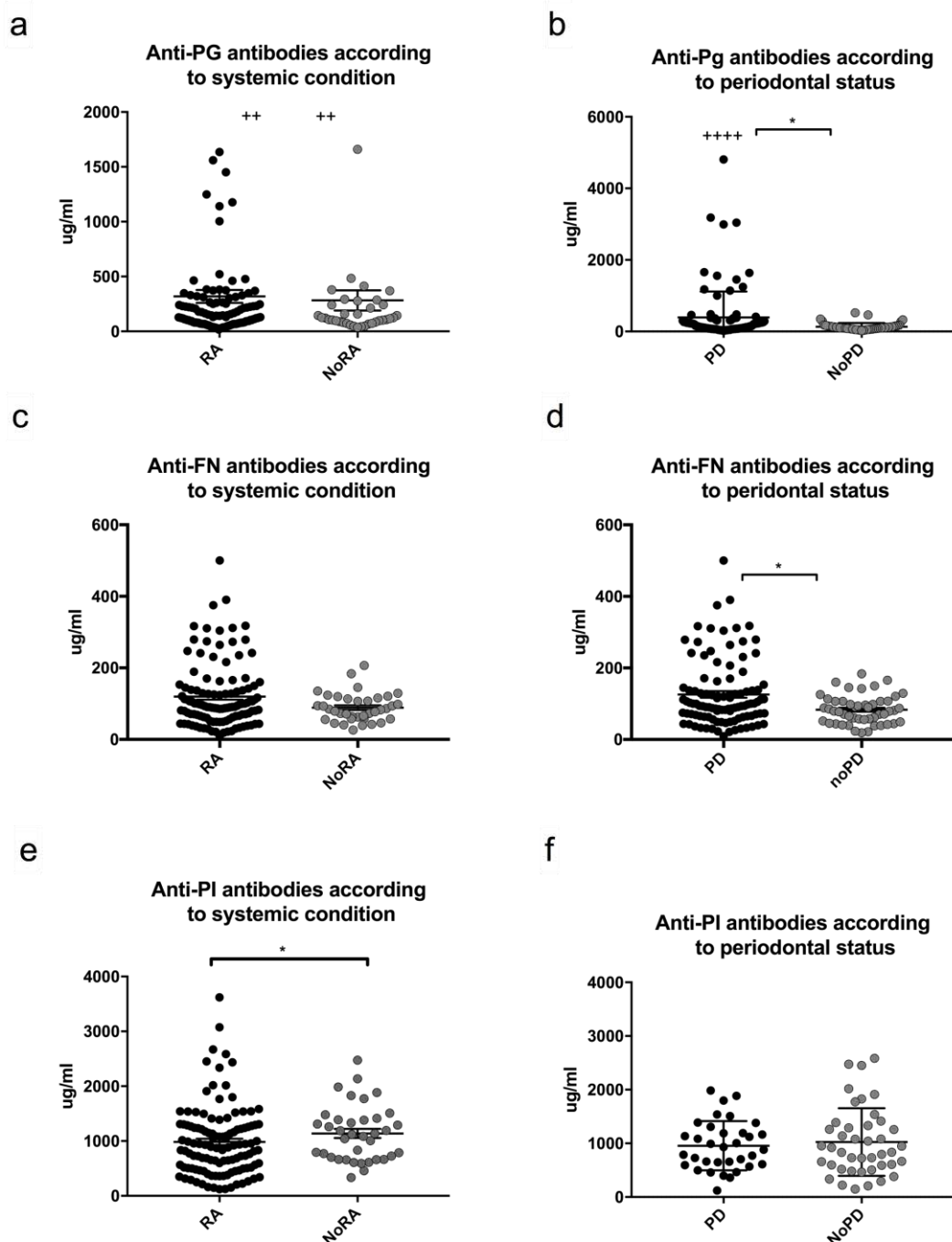


Figure 4. 8 Antibodies to OMAs from PG, FN and PI comparison according to periodontal or RA status. Mann-Whitney test, significance  $p < 0.05$ . N RA=143; n noRA=37. Data represented in ug/ml as scatter plot graphs and colored lines show the group mean and SEM. Each individual graph represents the results for each OMA: a. *Porphyromonas gingivalis* b., *Prevotella intermedia*, c. *Fusobacterium nucleatum*; Significant differences between groups were analysed using the Man-Whitney's test (singinificant if  $p < 0.05$ , + symbol represents an outlier). Methods described in section 2.3. Abbreviations: PG, *Porphyromonas gingivalis*; PI, *Prevotella intermedia*; FN, *Fusobacterium nucleatum*; RA, rheumatoid arthritis; PD, periodontitis.

#### 4.3.3. Antibody levels comparison subgroup analysis

Further analysis was conducted to elucidate differences in the antibody repertoire against bacterial proteins depending on the degrees of periodontitis, from mild to severe, in both RA and noRA groups. Results showed a high level of antibodies

to PI in the three periodontitis groups (

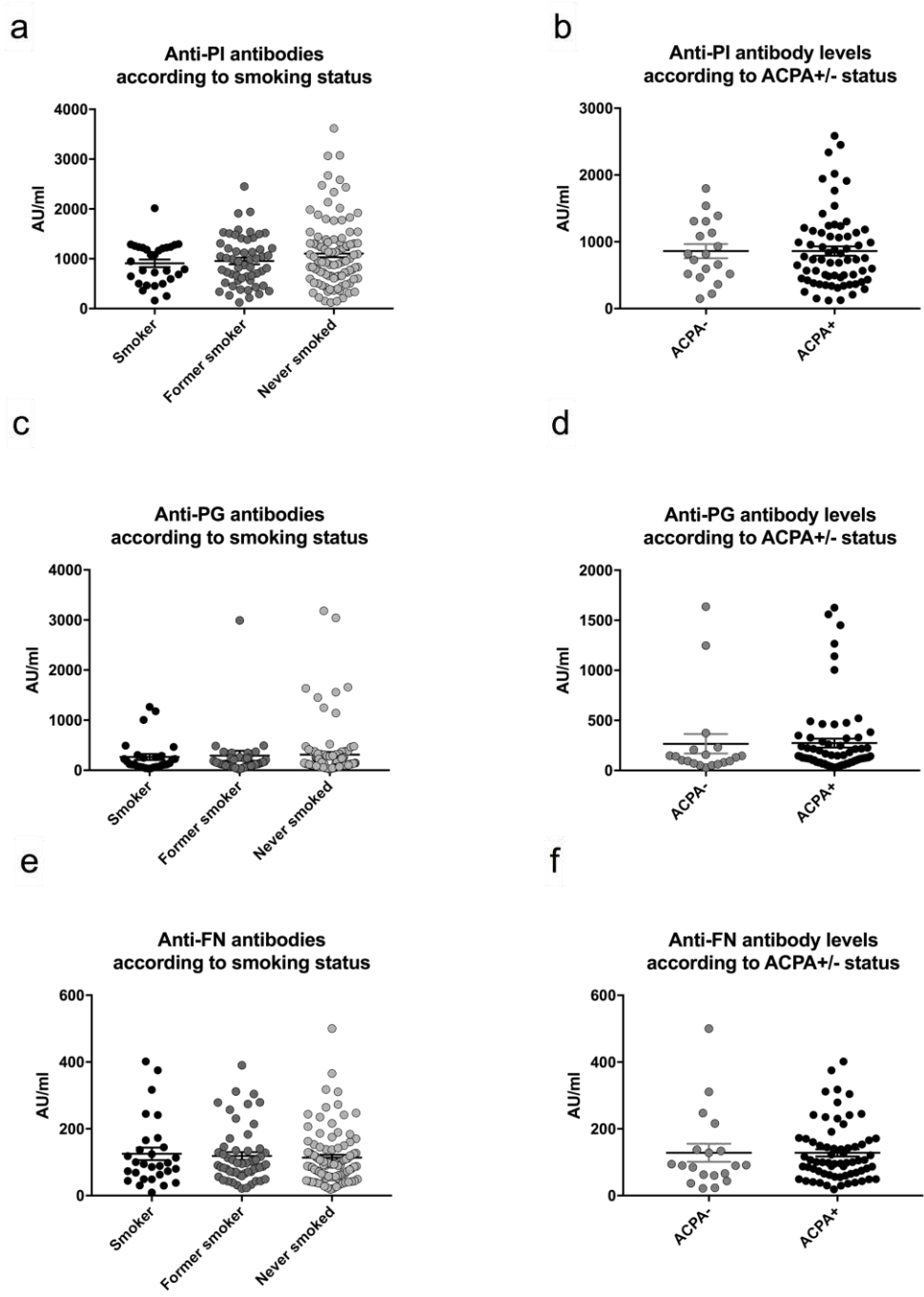


Figure 4. 11) also high in the periodontally healthy groups in the previous section. Much lower levels of antibodies are shown against FN, with no differences according to degrees of periodontitis. However, a significant increase of



antibodies against PG was observed as the severity of PD increases (ANOVA test  $p=0.048$ ). However, in the subgroup analysis according to systemic condition (RA vs no RA) or smoking status, no differences were found in the antibody levels to PG, FN or PI among the different groups (Figure 4. 9)

ACPA positivity is independently associated to periodontitis and to antibodies to PG (Mikuls, Payne et al. 2014) and anti-PG levels are higher in ACPA-positive patients (Hitchon, Chandad et al. 2010). However, in our study, no differences were found in antibodies to periodontal bacteria between ACPA positive and negative patients (i.e. anti-CCP levels  $>98^{\text{th}}$  percentile of health) in RA. Also an elevated antibody response to periodontal bacteria has been described to be associated with smoking status (Fisher, Cartwright et al. 2015), but this was not observed in our study for any of the bacterial OMA studied (Figure 4. 11).

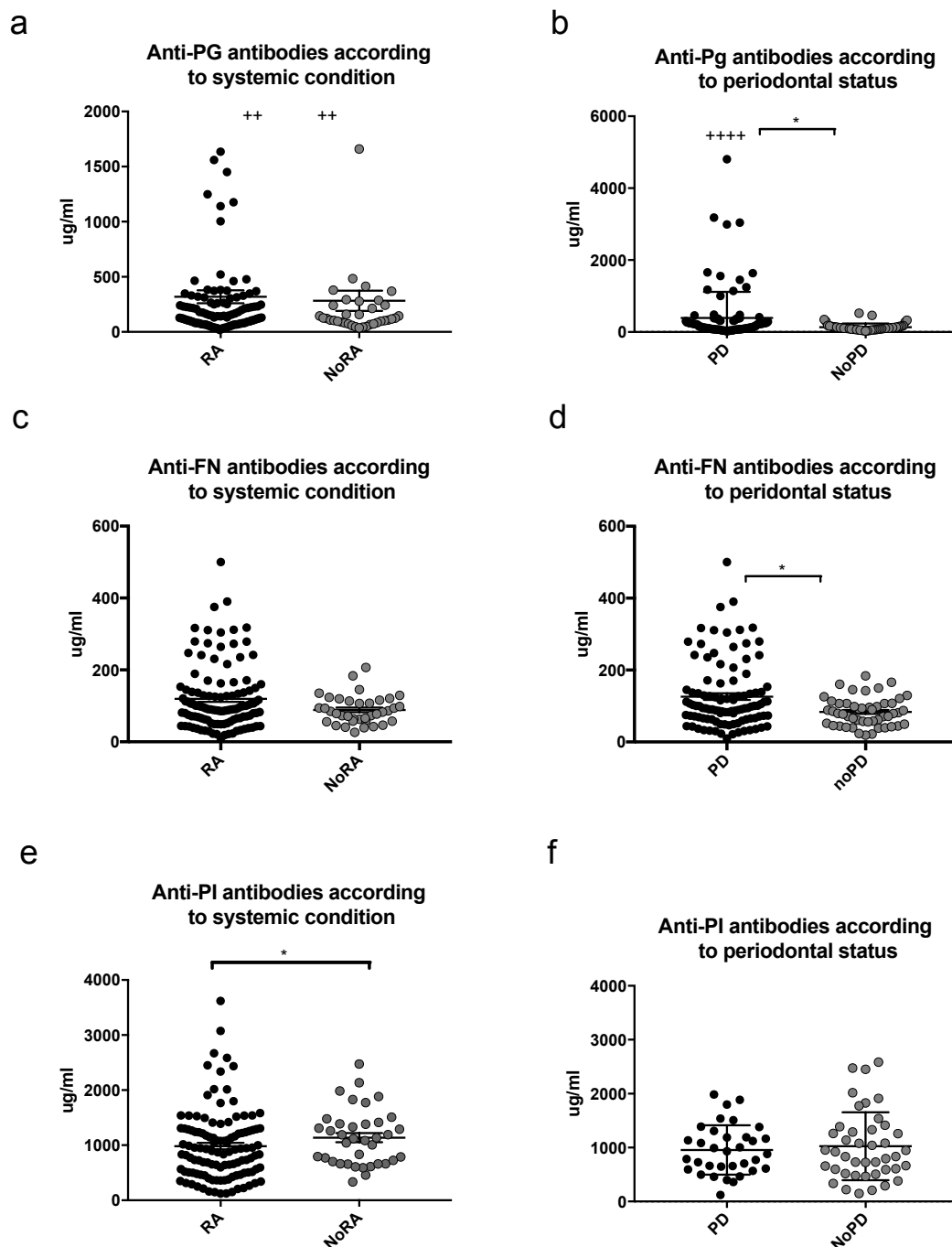


Figure 4. 9 Effect of periodontitis and systemic condition in levels of antibodies to PG, PI and FN in RA and noRA periodontitis patients. Kruskal-Wallis analysis was tested for the 3 bacteria individually with Dunn's multiple comparison (significance:  $p < 0.05$ ). Bar charts represent the mean with standard deviation of the mean. Abbreviations: PI, *Prevotella intermedia*; PG, *Porphyromonas gingivalis*; FN, *Fusobacterium nucleatum*; PD, chronic periodontitis; RA, rheumatoid arthritis.

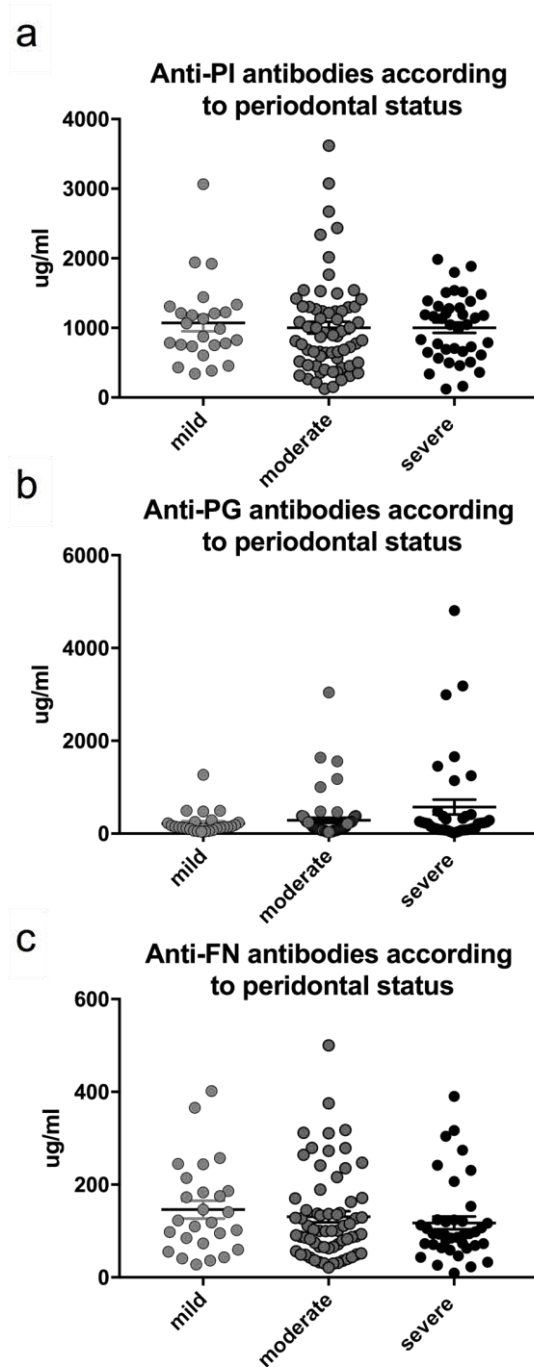


Figure 4. 10 Effect of severity of periodontitis in levels of antibodies to PG, PI and FN in RA and noRA periodontitis patients. Kruskal-Wallis analysis was tested for the 3 bacteria individually with Dunn's multiple comparison (significance:  $p < 0.05$ ). Bar charts represent the mean with standard deviation of the mean. Abbreviations: PI, *Prevotella intermedia*; PG, *Porphyromonas gingivalis*; FN, *Fusobacterium nucleatum*; PD, chronic periodontitis.

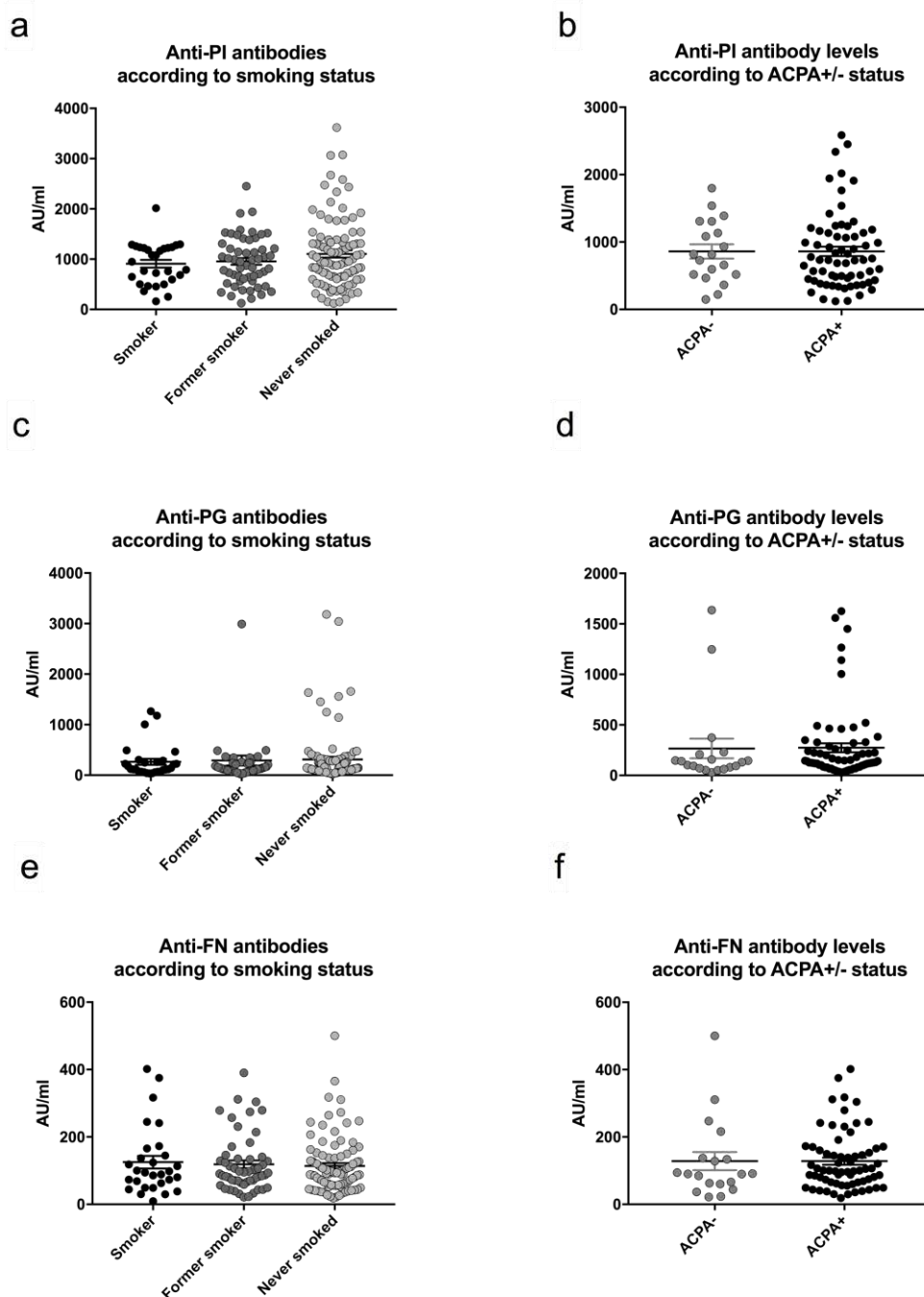


Figure 4. 11 Subgroup analysis of antibody levels according to smoking status and ACPA. N ACPA+= 68; n ACPA - = 19 ; n Smoker=29; n former smoker= 55, n never smoked=96. Statistical tests: Kruskal-Wallis with Dunn's test for multiple comparison and Mann-Whitney test for two group (significance:  $p < 0.05$ ). Abbreviations: PI, *Prevotella intermedia*; PG, *Porphyromonas gingivalis*; FN, *Fusobacterium nucleatum*; PD, chronic periodontitis.

#### **4.4. Longitudinal analysis**

##### **4.4.1. Patient background and effect of periodontal therapy on clinical characteristics**

The effect of periodontal therapy on clinical parameters and clinical characteristics of the immediate group compared to the delayed group are explained in Chapter 3 and the same study population was used in this chapter (n=42).

##### **4.4.2. Effect of periodontal therapy on PG-related antibody levels**

The longitudinal analysis of the antibody levels against PG-antigens showed no clear effect of periodontal therapy and none of the antibodies evaluated showed a statistically significant change between levels at baseline (V1) and 6 months after (V2) in the treatment or delayed group (Wilcoxon paired test, significance  $p < 0.05$ ). The experiment that demonstrated the closest p value to significance was the change in antibody response to PPAD in the treatment group ( $p = 0.06$ ), which showed a decrease in the antibody levels after periodontal therapy. While there was a slight reduction of antibody levels to RgpB and PPAD (Figure 4. 12), it is difficult to see a pattern. On the contrary of the trend observed for anti-human citrullinated proteins (chapter 3), there was no increase of antibody levels after periodontal therapy for any of the PG-related antigens studied, but there was no clear effect on the CPP/RPP3 and PG-enolase antibody levels. While a majority

of patients showed a small or no variation of antibodies to these PG-antigens after 6 months in both groups, a few patients experience big variations towards both increase and decrease. Subgroup analysis stratifying in ACPA status did not show a reduction in antibodies to PG-related antigens after periodontal therapy.

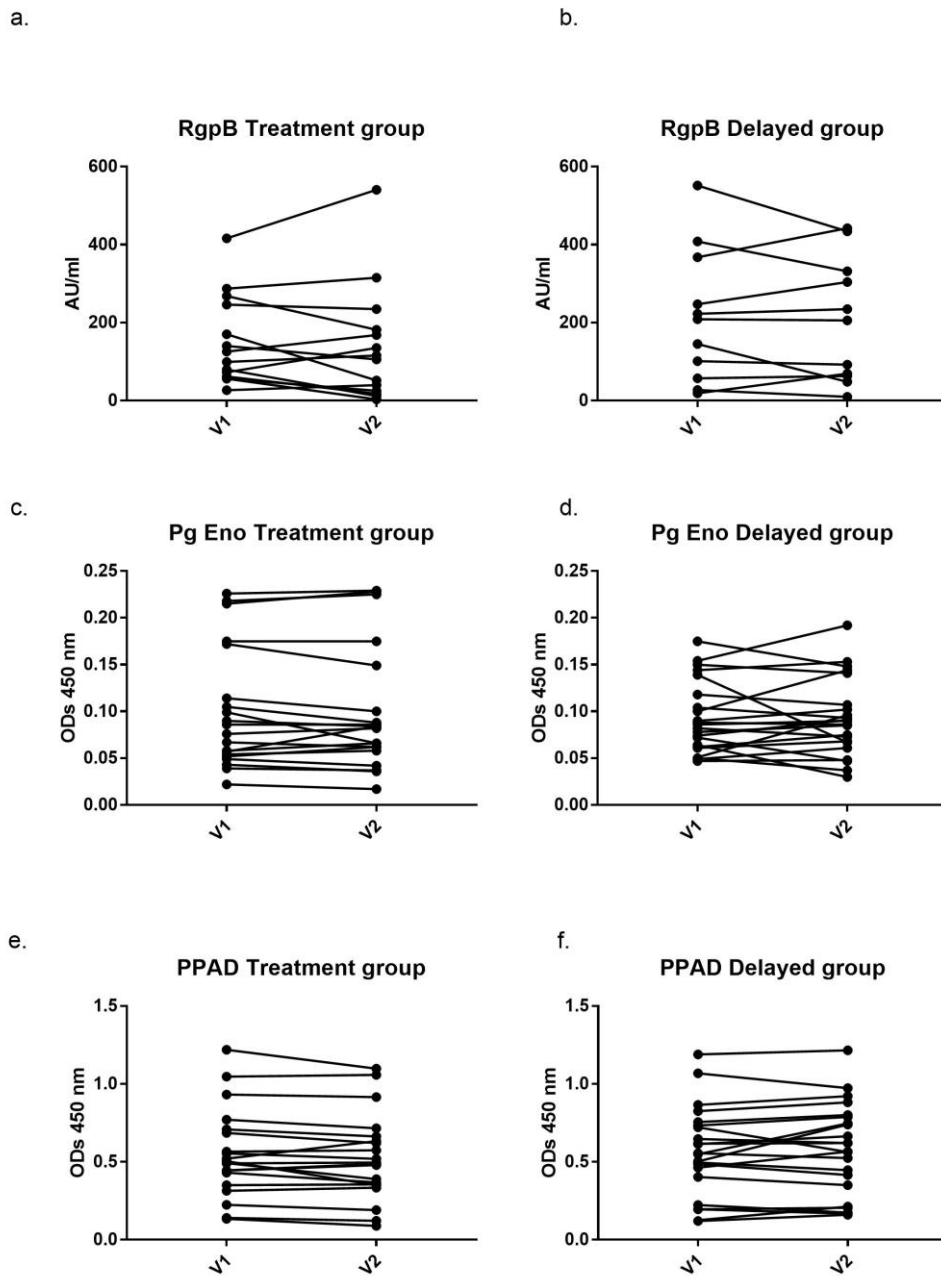
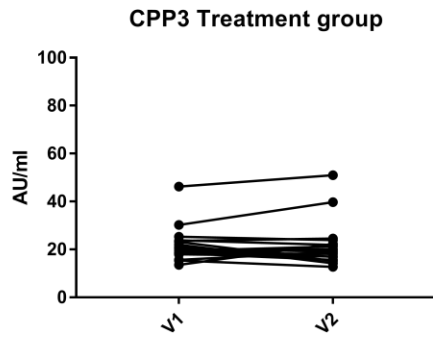
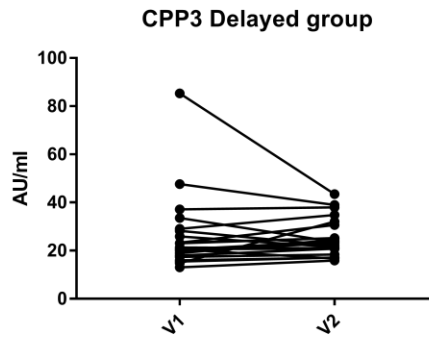


Figure 4. 12 **Longitudinal analysis of antibody levels to PG-related antigens;** a. and b. arginine gingipain; b. and c. PG-enolase; d and e. PG-peptidyl arginine deiminase. n Treatment group=20; n Delayed group=22. Graphs represent V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after treatment in the immediate group and hygiene instructions in the delayed group. Statistical test used was Wilcoxon matched pair test (significance “\*” p<0.05). Statistical test used was Wilcoxon matched pair test (significance p<0.05). Methods described in section 2.2.

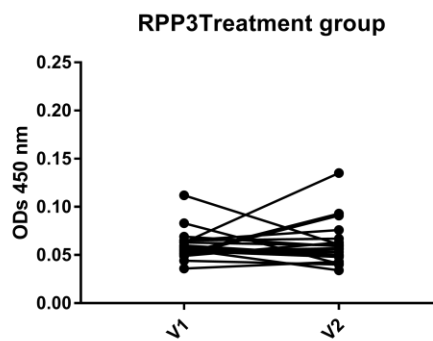
a.



b.



c.



d.

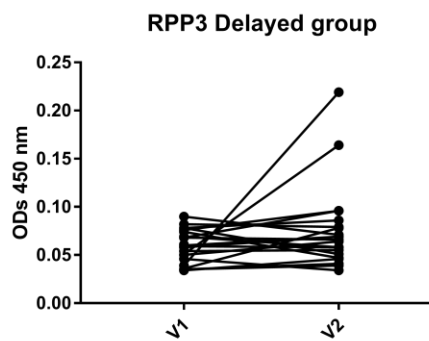


Figure 4. 13 Longitudinal analysis of antibody levels to citrullinated (a. and b.) and arginated (c. and d.) peptide from PPAD. n Treatment group=20; n Delayed group=22. Graphs represent V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after treatment in the immediate group and hygiene instructions in the delayed group. Statistical test used was Wilcoxon matched pair test (significance “\*”  $p < 0.05$ ). Methods described in section 2.2.



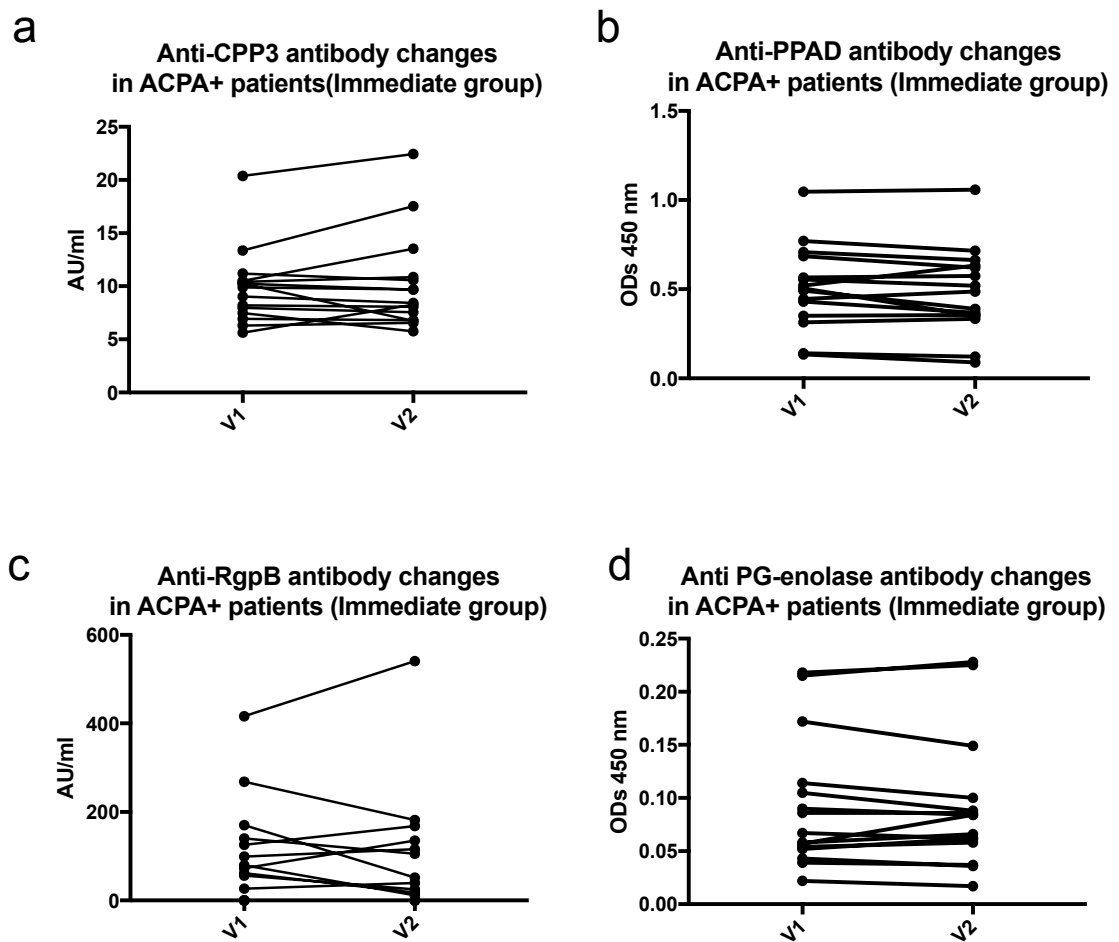


Figure 4. 14 Effect of periodontal treatment in antibody levels to PG related antigens in ACPA positive patients. N=15. V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after treatment in the immediate group. Statistical test used was Wilcoxon matched pair test (significance  $p < 0.05$ ). Methods described in section 2.2.

#### 4.4.3. Effect of periodontal therapy in OMAs antibodies

Periodontal therapy led to a reduction of antibodies to OMAs (Figure 4. 15) that was significant in the anti-FN levels (mean -14.92,  $p=0.026$ ) and not significant in anti-PI (mean -4.52,  $p=0.9$ ) and PG (mean -85,  $p=0.25$ ).

However, although no significance changes were found in the anti-PG antibodies, it can be observed that while the majority of patients had antibody levels close to zero, the patients with higher antibody reaction all underwent a reduction of antibodies after periodontal therapy that is not observed in the delayed group (Figure 4. 15. a). This pattern is not apparent in the antibody levels against PI and against FN. Although the last case showed significant changes, the changes seem rather small for the majority of patients (Figure 4. 15.e and f).

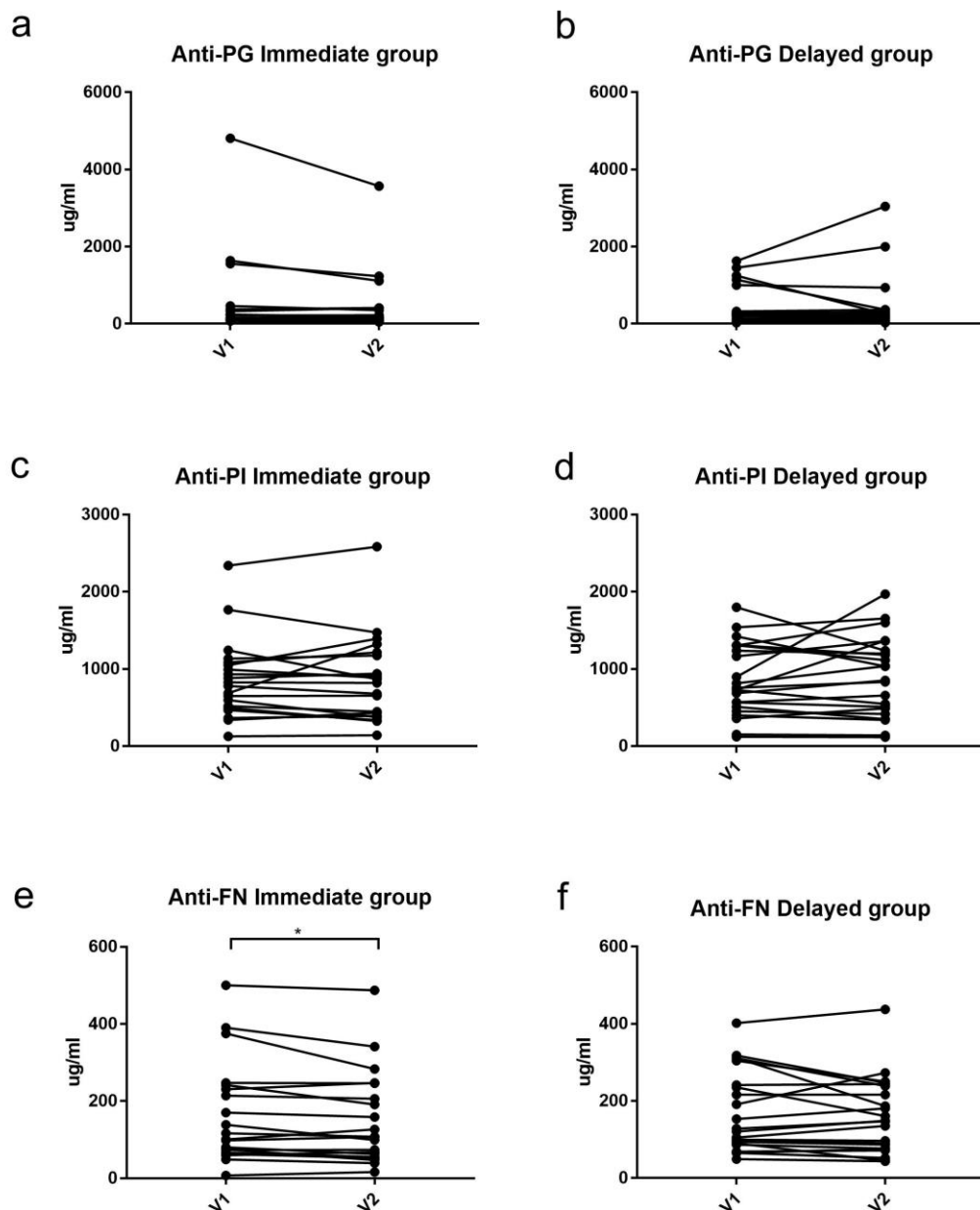


Figure 4. 15 Changes in antibodies to outer membrane antigens (OMA) from periodontal bacteria. Graphs represent changes in antibody levels (ug/ml) to OMAs from *Porphyromonas gingivalis* (PG), *Prevotella intermedia* (PI) and *Fusobacterium nucleatum* (FN) Treatment group=20; n Delayed group=22. V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after treatment in the immediate group and hygiene instructions in the delayed group. Statistical test used was Wilcoxon matched pair test (significance “\*” p<0.05). Methods described in section 2.3.

## 4.5. Correlation analysis

### 4.5.1. Clinical parameters

Periodontal and RA parameters were strongly and significantly correlated. Probing depth and CAL were significantly correlated with DAS28, ESR, number of swollen joints, number of tender joints and visual analogue scale related to RA activity (Figure 4. 16).

Correlation analysis on the changes between V1 and V2 was performed to investigate whether there is a correlation between the reduction of periodontal and rheumatological parameters and the changes in antibody production. This analysis showed a positive correlation between BoP and antibody response to PPAD ( $p=0.009$ , spearman's correlation= $0.42$ ) and negative correlation with antibodies to C-TNC5 ( $p=0.008$ , spearman's correlation= $0.439$ ). Although, at baseline, strong correlations were found between RA parameters and periodontal parameters, no correlation was found between changes on these parameters.

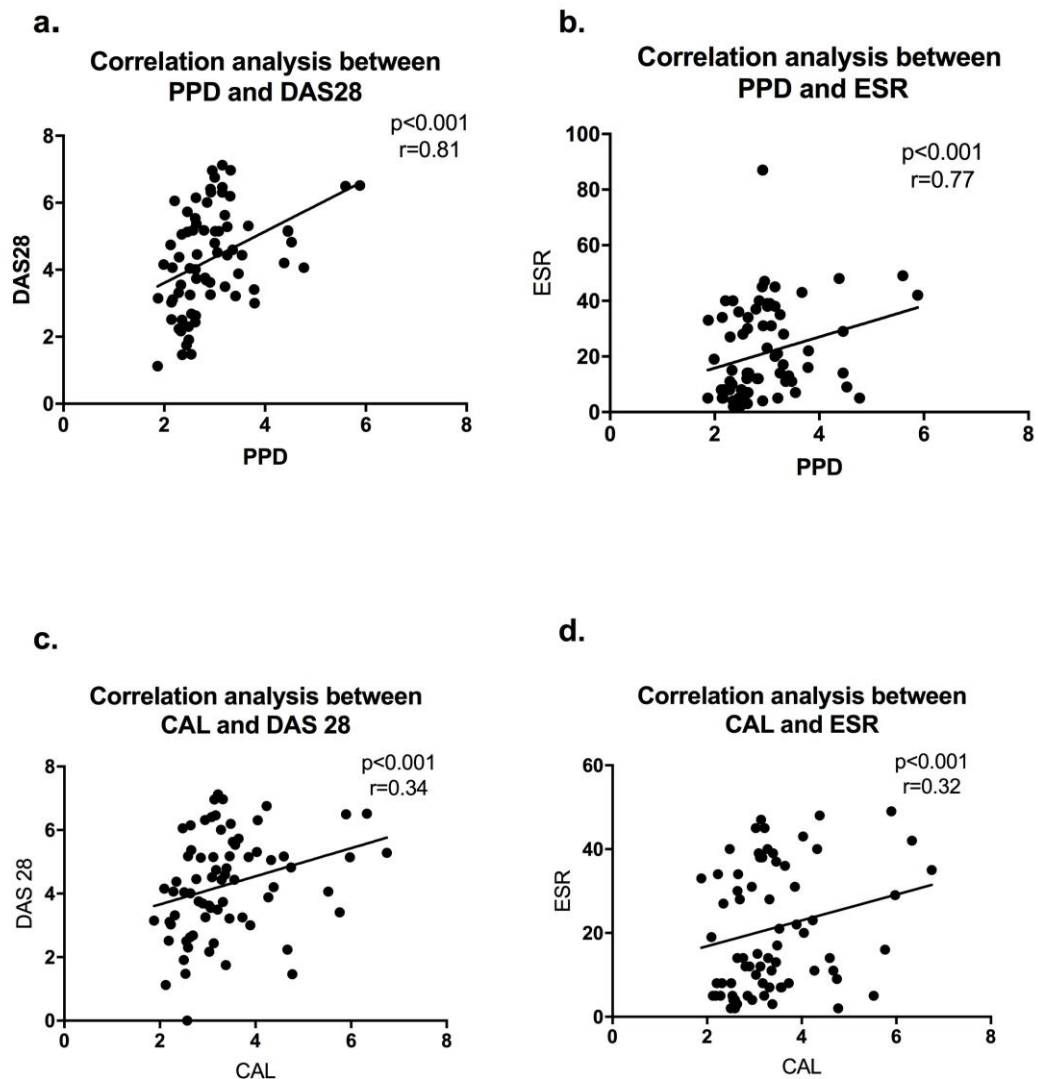


Figure 4. 16 Correlation analysis between periodontal and RA clinical parameters. Spearman's correlation (significance  $p < 0.05$ ). Abbreviations: CAL, clinical attachment loss; DAS28, disease activity score of 28 joints; ESR, erythrocyte sedimentation rate.

#### 4.5.2. Correlation analysis of antibodies to bacterial proteins and antibodies to citrullinated proteins

Weak significant correlations were found between anti-OMAs to PG antibodies to citrullinated proteins. Antibody levels against PG were positively correlated with antibodies to C-TNC5 ( $R=0.287$ ,  $p=0.002$ ), CCP ( $R=0.215$ ,  $p=0.023$ ). Anti-OMAs to FN and PI were not correlated with any of the antibodies to citrullinated or arginated peptides tested. As expected, Anti-PG positively correlated with anti-PPAD ( $R=0.196$ ,  $p=0.032$ ) and anti-RgpB ( $R=0.359$ ,  $p<0.001$ ).

From the correlation analysis between the anti-OMAs to the 3 periodontal bacteria, only PI and PG significantly correlated ( $r=0.2$ ,  $p=0.02$ ) although antibody levels to PI were negatively correlated to PPAD ( $R=-0.208$ ,  $p=0.023$ ). Antibody levels to FN were positively correlated to PG-eno ( $R=0.420$ ,  $p<0.001$ ) and PPAD ( $R=0.302$ ,  $p=0.001$ ).

#### 4.5.3. Correlation between antibodies to periodontal bacteria and clinical parameters

Antibodies to OMA from PG were significantly and positively correlated to periodontal parameters such as cumulative pocket depth ( $R=0.288$ ,  $p=0.002$ ), bleeding on probing ( $R=0.217$ ,  $R=0.019$ ), and CAL ( $0.32$ ,  $p<0.001$ ) (Figure 4. 17).

Antibodies to FN were weakly but significantly positively correlated to some periodontitis parameters, such as pocket depth ( $R=0.288$ ,  $p=0.002$ ) and bleeding on probing ( $R=0.193$ ,  $p=0.039$ ) and also some of the RA parameters, such as DAS 28 ( $R=0.237$ ,  $p=0.01$ ) and ESR ( $R=0.239$ ,  $p=0.01$ ) (Figure 4.16). However, anti-PI was weakly negatively correlated to all the DAS28 ( $R=-0.91$ ,  $p=0.034$ ) and the parameters used for the DAS calculation and even age ( $R=-0.394$ ,  $p<0.001$ ).

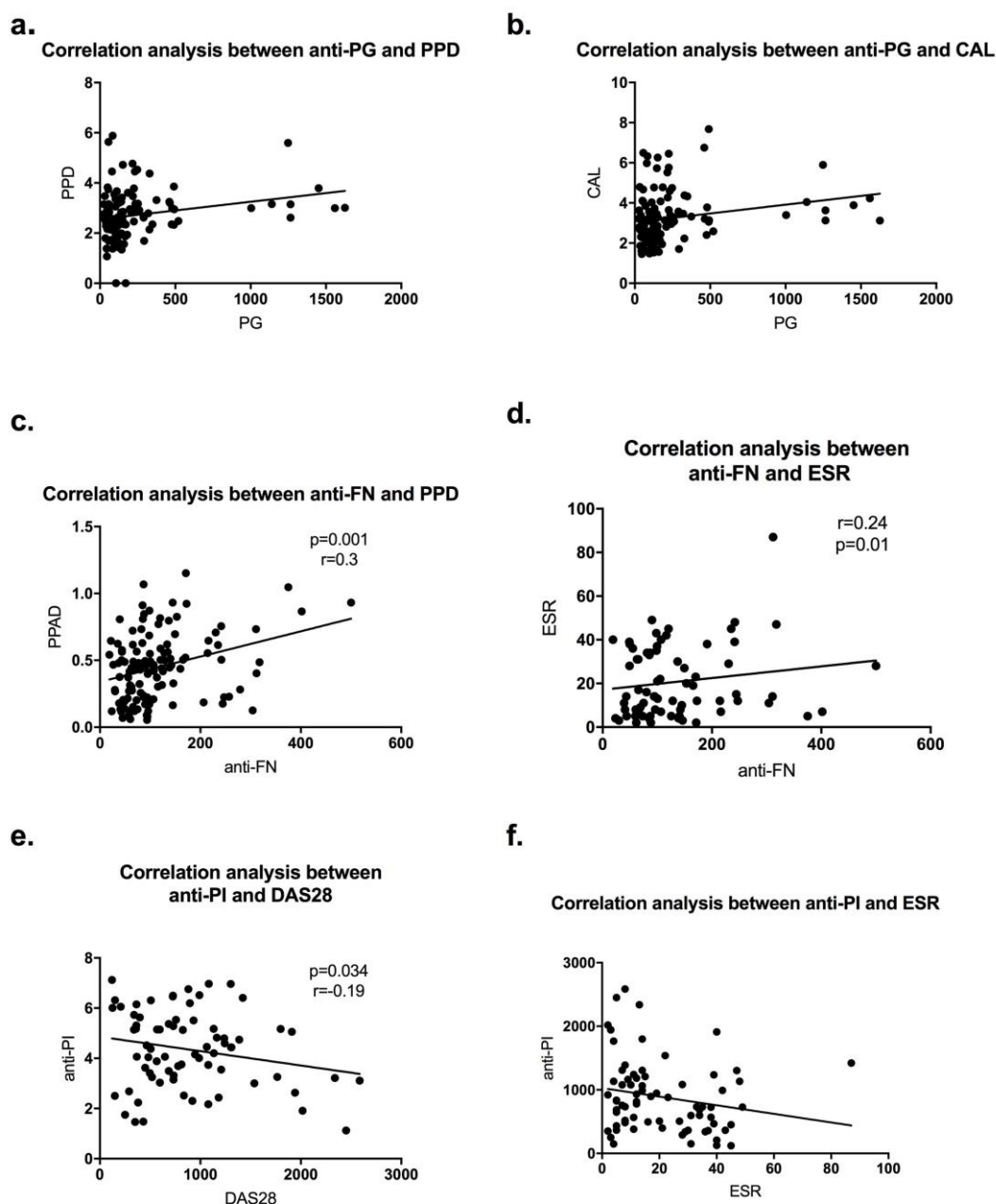


Figure 4. 17 Correlation analysis between OMA antibodies to PG, FN and PI (AU/ml), and clinical parameters. Test: spearman's correlation (significance:  $p < 0.05$ ). Abbreviations: PG, *Porphyromonas gingivalis*; PI, *Prevotella intermedia*; FN, *Fusobacterium nucleatum*. PPD, probing pocket depth; CAL, clinical attachment loss; DAS28, disease activity score of 28 joints; ESR, erythrocyte sedimentation rate.



#### 4.5.4. Correlation between serum antibodies to PI, PG and FN and their relative abundance in subgingival plaque

The relative abundance of these 3 bacteria (*Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacteria nucleatum*) investigated with 16s rRNA methods (section 2) showed no significant change after periodontal therapy in the immediate group. While PG and FN were non-significantly reduced, PI was increased (Table 4. 2). However, this did not necessarily reflect the amount of bacteria present in the mouth of the patients, as after periodontal therapy, it has been described that there is a profound reduction of counts of both supra and sub-gingival bacteria (Ximénez-Fyvie, Haffajee et al. 2000). As plaque score was not recorded in this study, the plaque reduction as a result of periodontal therapy cannot be confirmed and only the relative abundance could be analysed, showing no significant differences in the relative abundance of PI, PG or FN after periodontal therapy.

Spearman's correlation analysis was performed between the levels of antibody to membrane proteins from PG, PN and FN in serum and the relative abundance of these bacteria in subgingival plaque (Figure 4. 18). This analysis was performed in the data from all patients that had both types of samples analysed (N=148), from the 4 baseline groups (RAPD, RA noPD, NoRA PD, NoRA noPD). Levels of anti-PG antibodies correlated with the relative abundance of PG, FN and PI ( $p < 0.05$ ). The most significant and strongest correlation found was

between anti-PG antibodies and relative abundance of PG ( $R=0.33$ ,  $p<0.0001$ ). However, no correlation was found between antibodies to the other 2 bacteria and their corresponding relative abundance.

Results showed that levels of the antibody against proteins from the 3 bacteria are significantly and positively correlated with each other, however, these correlations were weak ( $r<0.4$ ).

Table 4. 2 Results of significance test between relative abundance of PG, PI, FN after periodontal therapy (Immediate group) after 6 months. Test: Wilcoxon rank test for related samples.

	N	Mean difference of relative abundance (baseline and 6 months)	P value (paired T-Test)
<i>Prevotella intermedia</i>	18	0.005	0.91
<i>Porphyromonas gingivalis</i>	18	-0.007	0.27
<i>Fusobacterium nucleatum</i>	18	-0.024	0.22

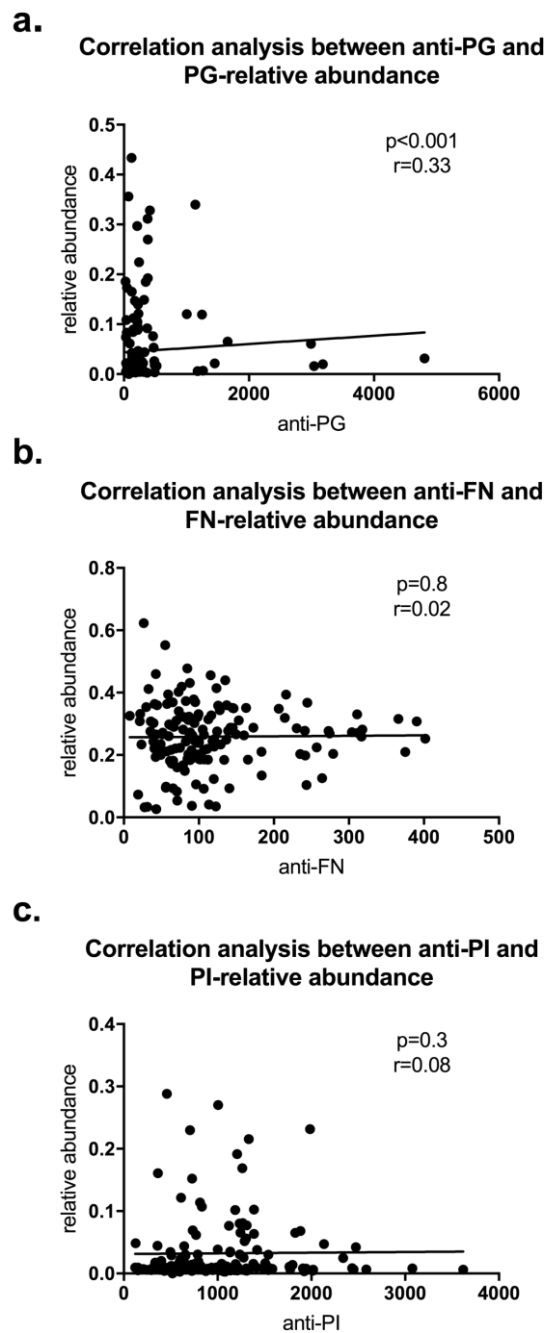


Figure 4. 18 Correlation analysis between serum antibodies to OMA antibodies to PG, FN and PI (AU/ml), and their relative abundance in subgingival plaque. Test: spearman's correlation (significance:  $p < 0.05$ ).  $N = 148$ . Abbreviations: PG, *Porphyromonas gingivalis*; PI, *Prevotella intermedia*; FN, *Fusobacterium nucleatum*; N, number of patients investigated.

#### 4.5.5. Correlation between the changes produced after periodontal treatment

Spearman's correlation analysis was performed to investigate whether the changes in antibody response to periodontal bacteria after periodontal treatment were associated to the changes on the relative abundance to these bacteria. The non-significant reduction in relative abundance of PG and FN after periodontal therapy was positively correlated in the immediate group ( $R=0.613$ ,  $p=0.009$ ) and also in the delayed group data. Changes in antibody levels to PG were also correlated to the relative abundance of FN in the immediate group ( $R=0.613$ ,  $p=0.009$ ) and PI ( $R=0.458$ ,  $p=0.049$ ) and antibodies to PPAD and anti-PG OMAs were correlated ( $R=0.672$ ,  $p=0.003$ ).

In the delayed group the changes in antibodies to PG and anti-FN were significantly correlated ( $R=0.68$ ,  $p<0.001$ ) but the significant correlation between the change in relative abundance of FN and PI ( $R=0.53$ ,  $p=0.012$ ) in the immediate group did not appear in the delayed group.

#### 4.6. Overlap of antibody reactivity

In RA, clustering of antibodies has been previously studied using a heatmap to compare pattern of autoantibody reactivities (Wegner, Wait et al. 2010). In our study, no clear patterns were observed in the antibody reactivity to PG-related antigens as a small group of patients were positive for the antibodies studied

(>95th percentile of healthy controls), regardless of periodontal or ACPA status (Figure 4. 19).

Although the antibody levels to PG-related antigens are significantly elevated in RA compared to controls, the percentage of RA patients with antibody levels over the 95th percentile of healthy control was low for all the PG-antigens. 25 % of RA patients were antibody negative for all the antigens and no patient revealed antibodies to all the bacterial antigens studied, with a majority of patients (63%) being antibody positive to one or two antigens only. However, in the small group of anti-CCP negative patients, we see a majority of patients (61%) with antibodies to one or more of the bacterial antigens investigated, from both periodontally healthy and periodontitis groups. This shows that the antibody positivity against PG-related antigens was not only due to cross-reactivity with ACPAs, as patients with low detection of antibodies to citrullinated antigens can have high levels of antibodies to PG-related antigens (Figure 4. 19).

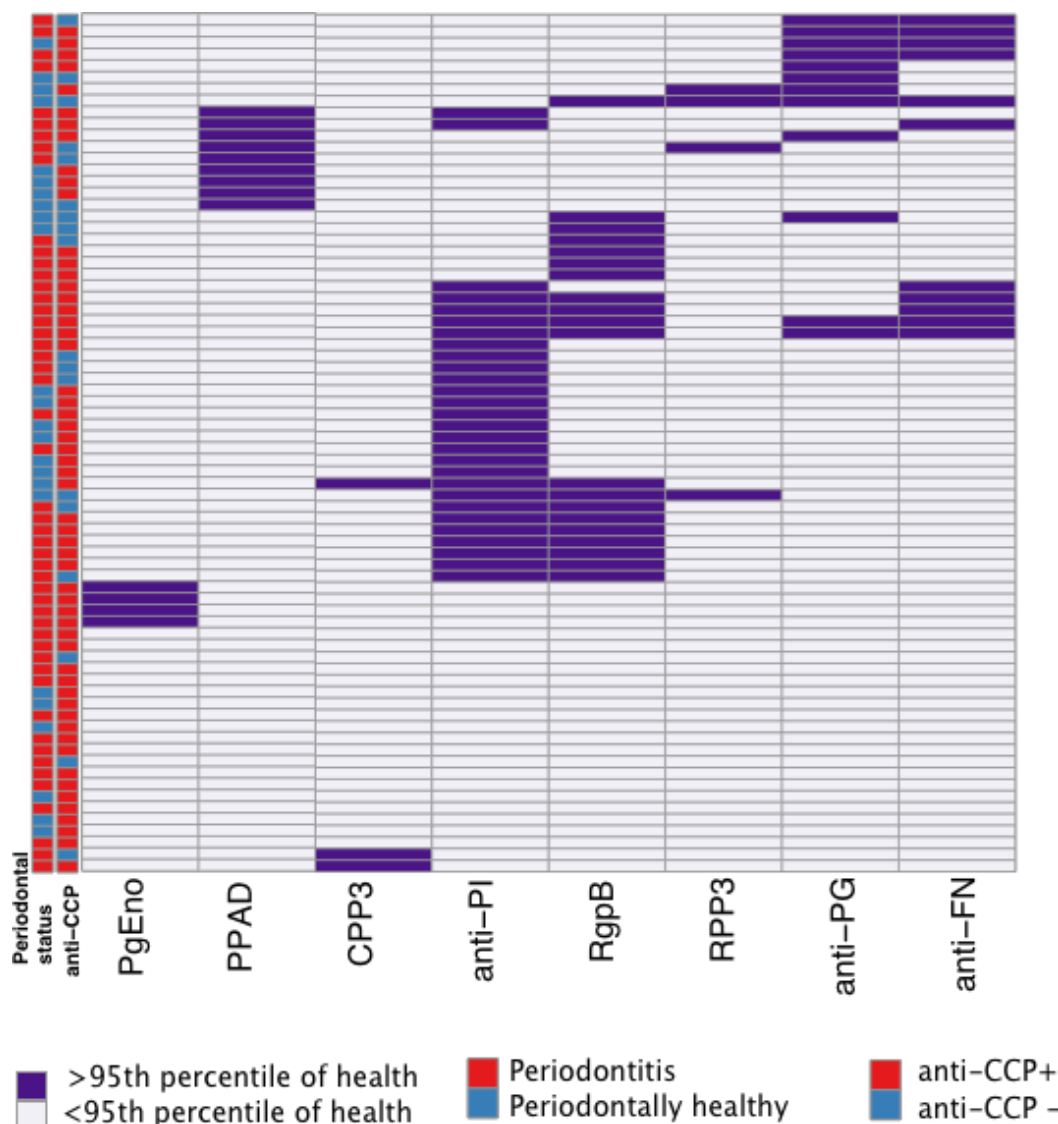


Figure 4. 19 Heatmap of RA patients showing antibody positivity (>95th percentile of healthy controls for each antibody titre) in all antibodies to periodontal bacteria studied classified according to anti-CCP positivity and presence of periodontitis. Each row represents one patient. The color scale shows the relative degree of antibody reactivity, from low reactivity (white) to high reactivity (purple). The order of the columns is according to their relationship with each other and anti-CCP. Abbreviations: PG, Porphyromonas gingivalis; PPAD, PG peptidylarginine deiminase; CPP3/RPP3, citrullinated and arginated epitope from PPAD; Pg-eno, enolase from PG; RgpB, *P. gingivalis* virulence factor arginine gingipainB; CCP, cyclic citrullinated peptid; PI, *Prevotella intermedia*; FN, *Fusobacterium nucleatum*.

## 4.7. Discussion

### 4.7.1. Anti-PG Crossectional comparison

The study of antibodies against different antigens from Pg is of special importance, not only to elucidate the role of Pg in RA but because it has been suggested that antibodies against Pg are associated to non-respondent reactions to DMARDs in RA (Kobayashi, Ito et al. 2016). The hypothesis of the cross-sectional study comparison was that patients with RA have elevated antibodies to periodontal bacteria, which was corroborated by our results. RA patients had significantly higher levels of antibodies against PG-antigens (Figure 4. 3). This antibody reaction, occurred in both periodontitis and periodontally healthy groups, however did not occur in anti-CCP negative patients who were periodontally healthy (Figure 4. 6). These findings support the hypothesis that, in RA, PG triggers an immune reaction that, by epitope spreading, becomes an auto-antibody reaction against citrullinated proteins in the joint.

Our results suggest that, PPAD and its epitopes (CPP3 and RPP3), act as an antigen in RA but not RgpB, which is targeted mostly in periodontitis patients (Figure 4. 1). These findings are in accordance with those from Quirke et al (Quirke, Lugli et al. 2014) and Johansson et al. (Johansson, Sherina et al. 2016) whose papers show that RgpB and CPP3 antibody levels follow different trends, being anti-RgpB antibodies elevated in PD but not RA, and anti-PPAD antibodies elevated in RA.

It could be argued that the anti-CPP3 antibody response in RA, rather than being *P. gingivalis* specific, could simply belong to the generic ACPA response, or rather represents cross-reactivity with another citrullinated antigen. However, the non-citrullinated epitope from PPAD, RPP3, was also elevated in RA noPD compared to NoRA PD, which would not correspond to a typical ACPA response. Although these differences in RPP3 could be questioned, as the ODs detected were low for all patients and no standard curve could be selected, the heatmap of the anti-PG antigens and anti-CCP positivity (Figure 4. 19) showed no patterns between those patients that were anti-CCP positive and the patients that were anti-PG positive and a majority of patients anti-CCP negative were antibody positive to one or more PG-related antigens. Considering all this, it could be suggested that the antibody reaction against PG observed in this study was not just due to cross-reactivity but an antibody response significantly enhanced in RA.

The lack of differences found in antibody levels to PG-antigens according to smoking status contradicts previous findings (Mikuls, Payne et al. 2014) but this could be explained due to the small number of smokers in our study (Figure 4. 5). The elevated antibody reaction in the moderate to severe periodontitis group was not unexpected as there was also an elevated abundance of PG in these patients (Figure 4. 4).

#### 4.7.2. OMAs cross-sectional comparison



The identification of the outer membrane antigens (OMA) studied in the study was accomplished in a periodontitis case-control by Curtis and colleagues (Curtis, Slaney et al. 1991). Following the methods previously described by Mikuls and collaborators (Mikuls, Payne et al. 2014), antibodies to OMA from PG, were studied in this chapter as it has been hypothesized that these are increased in RA, and antibodies to FN and PI were also analysed as both bacteria are closely associated with PG in the deep pocket biofilm (Holt and Ebersole 2005).

*Porphyromonas gingivalis* manipulates the immune system by different mechanisms to perpetuate inflammation with the help of other periodontal bacteria from the so-called “orange complex” or 2<sup>nd</sup> group, which includes PI and FN (Socransky, Haffajee et al. 1998). *Prevotella intermedia* can evade immunity through degradation of C3 and other key components of the complement, by capturing complement factor H and by inhibit complement and it is associated with deep periodontal pockets with bleeding on probing (Zambon 1996). However, in our study, antibodies to PI seemed to be associated with health instead of disease. This could represent a healthy protective antibody response that is reduced in disease.

*Fusobacterium nucleatum* is a Gram-negative bacillus that acts as an opportunistic pathogen in periodontal disease, breaking through the gingival epithelium and interfering with the host defences (suppression of ROS release and B-defensine expression) and fibroblast proliferation and it facilitates the colonization of other periodontal pathogens. It is also related to adverse

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pregnancy outcomes. FN has been found to be in high concentrations in RA patients compared to controls (Schmickler, Rupprecht et al. 2017). In our study FN was significantly elevated in the RAPD group compared to the RA<sub>no</sub>PD group and there was a clear large group in the RAPD with high levels of anti-FN compared to the rest of the groups, and also in the systemically healthy group with periodontitis. The conditions of both RA and PD seemed to favour the growth of FN, as its relative abundance and antibody response was elevated in these groups, and periodontal therapy seemed to significantly reduce the load of this bacteria in the periodontal pocket.

There is a clear correlation between periodontitis and high levels of antibodies to *P. gingivalis* in both RA or noRA groups. This bacterium showed the strongest correlation to periodontitis of the ones studied here, in agreement with the theory of Socransky et al., which describes *P. gingivalis* as a red complex bacteria, closely associated with severe periodontitis. However, neither the relative abundance of *P. gingivalis* or the antibodies to *P. gingivalis* were significantly elevated in RA patients compared to control, and antibodies to any of the PG-related peptides or OMAs were not significantly reduced after periodontal therapy. Although the relative abundance of *P. gingivalis* was not reduced after periodontal therapy, the amount of *P. gingivalis* present in the oral cavity of the patients that were treated may be reduced as the amount of plaque was reduced.

The lack of a reduction in the immune reaction against *P. gingivalis* could mean that, while in the initial stages of RA, *P. gingivalis* is still a possible candidate to

trigger the autoimmune reaction that leads to ACPA formation, once RA is established, even if *P. gingivalis* is reduced by periodontal therapy, the autoimmune reaction that occurs in the joint perseveres.

#### 4.7.3. Correlation analysis

In our study, there were significant associations between antibodies to *P. gingivalis* and OMAs from FN and clinical parameters (Appendices Table 8. 1). While antibodies to FN seemed to be associated with both RA and periodontitis parameters, anti-PG antibodies were mostly associated to periodontal parameters, also it was also significantly but weakly associated to anti-CCP antibodies. Mikuls and collaborators found similar weak but significant associations between OMAs to *P. gingivalis* and anti-CCP (Mikuls, Payne et al. 2014). Also Lappin and collaborators found correlations between anti-CCP, anti-PG and anti-CCP and CEP/REP. When smokers were included, the correlation between anti-CCP and anti-PG was negative, when smokers were excluded, the correlation was positive which the authors attributed to smoking acting as confounding factor, which was not seen in our study. This latter paper also observed a reduction of anti-PG and anti CCP after periodontal therapy, which contradicts our results, although they did not include RA patients (Lappin, Apatzidou et al. 2013). Lee found correlations between anti-enolase and anti-PG (Lee, Choi et al. 2015), however this correlations were similarly weak to the ones found in this study.

In our study, it was not observed higher anti-PG antibody levels in anti-CCP positive patients (Hitchon, Chandad et al. 2010). This might be due to the small number of anti-CCP negative patients included, resulting in a lack of power to detect significances between groups with very different numbers.

The significant correlation between anti-PG and relative abundance of *P. gingivalis* ( $R=0.33$ ,  $p<0.0001$ ) suggested that anti-PG OMAs could be used as an indicative parameter for the presence of *P. gingivalis*. Thus, considering this weak but consistent correlations through numerous publications, it could be stated that antibodies to citrullinated proteins are associated with anti-PG and therefore, the presence of *P. gingivalis*, strengthening the theory of *P. gingivalis* as being the driver of the antibody reaction in RA.

#### 4.7.4. Longitudinal study results

Our hypothesis, following the theory of extra-articular citrullination in periodontitis triggering and exacerbating RA, was that periodontal treatment could bring down levels of *P. gingivalis* and therefore, the production of antibodies against *P. gingivalis*. This is supported by previous research that found significant reduction of anti-PG after periodontal therapy (Okada, Kobayashi et al. 2013) (Lappin, Apatzidou et al. 2013).

Numerous studies have examined the changes in antibodies to periodontal bacteria after periodontal therapy. In the studies from Ebersole (Ebersole, Taubman et al. 1985) and Okamura (Murayama, Nagai et al. 1988), the authors

found an elevation of serum antibody levels to periodontal bacteria immediately after periodontal therapy, but after a few months, antibodies to PG and other periodontal bacteria seem to be reduced and correlated with clinical parameters (Horibe, Watanabe et al. 1995). Other authors defend that in Rapidly Progressive Periodontitis (RPP), periodontal therapy stimulates the production of high avidity antibodies to PG, replacing low avidity antibodies (Chen, Johnson et al. 1991). However, in our study, we did not find a significant effect of periodontal therapy on levels of antibodies to any of the human and PG-related peptides studied. Likewise, a recent study published by Shimada and collaborators failed to find a reduction in anti-CCP levels and anti PPAD in RA, 2 months after periodontal therapy (Shimada, Kobayashi et al. 2016). These negative results contradict those from Okada et al (Okada, Kobayashi et al. 2013), who found a significant decrease in antibodies to PG in RA in the treatment group compared to control. Nevertheless, in contrast with our results, Okada did not include periodontitis patients and only conducted supra gingival cleaning. These opposing results could be explained by differences in methodology, as the study from Okada used a mild definition of periodontitis and provided supragingival scaling, while the paper from Shimada and our paper included moderate to severe periodontitis patients and provided subgingival periodontal therapy. While it seems that in mild cases of periodontitis a simple supragingival scaling might be enough to reduce the level of *P. gingivalis* and therefore reduce citrullination, in periodontitis it might be more complex to demonstrate this effect. While supragingival cleaning seems to be beneficial in levels of citrullination and antibody response to *P. gingivalis*, in periodontitis patients with RA this decrease was not observed.



## **5. RESULTS – SUBGINGIVAL MICROBIOME IN RA**

## 5.1. Introduction

The hypothesis of the present chapter was that there is a cross-talk between the oral microbiome and the immune system in RA that produces changes in the microbiome of these patients compared to systemically healthy individuals. These changes could partially be restored by periodontal treatment.

The aims of this project were:

- 1) To characterize the subgingival microbiota of RA patients who are periodontally healthy compared to controls (RA no PD vs no RA no PD).
- 2) To characterize the subgingival microbiome of RA patients with periodontitis compared to the appropriate controls (RA no PD and No RA PD)
- 3) To investigate the effect of periodontal treatment in the subgingival microbiota of RA patients with periodontitis compared to controls (Delayed group) after 3 and 6 months.

Secondary aims were proposed in order to investigate differences in the oral microbiome, comparing groups such as systemically healthy with and without periodontitis, periodontitis microbiome from shallow sites vs deep sites and the effect of clinical parameters (smoking, age, ACPA status, etc). To these ends, the subgingival community was explored using 16S rRNA through Illumina MiSeq platform and different groups of patients were compared according to each research question (Figure 5. 1, Table 5. 1). Methods are described in chapter 2.5.



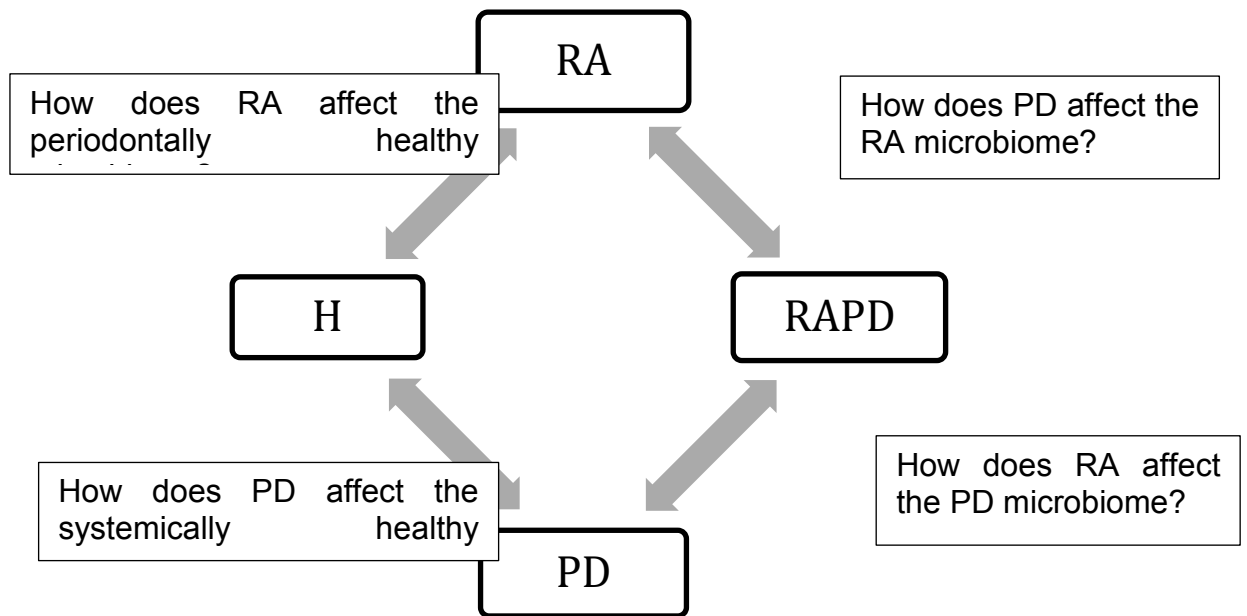


Figure 5. 1 Diagram of scientific questions of the cross-sectional study comparison (aims 1, 2 and subgroup analysis). Abbreviations: RA, rheumatoid arthritis; PD, periodontitis; H healthy controls. Methods described in section 2.5.

Table 5. 1 Different comparisons presented in this chapter, the section where investigated and aim as the effect of the different parameters in the oral microbiome.  
Abbreviations: RA, rheumatoid arthritis; PD, periodontitis.

<b>Group comparison</b>	<b>N</b>	<b>Section</b>	<b>Aim</b>
All patients (crosssectional)	160	5.2	Effect of each clinical parameter
+/-RA +/-PD	111	5.3	Effect of both RA and PD
RAnoPD vs noRAPD	41	5.4	Effect of RA in noPD
RAPD vs noRAPD		5.5	Effect of RA in PD
RAnoPD vs RAPD		5.6	Effect of PD in RA
Immediate group: V1 vs V2		5.7.2	Effect of periodontal therapy after 3 months
Immediate group: V1 vs V3		5.7.3	Effect of periodontal therapy after 6 months
Delayed group: V1 vs V2 vs V3		5.7.5	Changes after 3 and 6 months in control group
noRAPD vs noRAnoPD		5.8.1	Effect of PD in noRA
Shallow vs deep pockets		5.8.2	Effect of pocket selection
Curette vs paperpoint		5.8.4	Effect of method of collection
DMARDs vs DMARDS+biologics vs biologics		5.8.4	Effect of RA medication

## 5.2. Cross-sectional analysis of study population

### 5.2.1. Rarefaction analysis

Rarefaction analysis is used to assess the richness of the species as a function of number of samples, which plots a curve that grows rapidly as the common species are identified and plateau once the most rare species remain. To demonstrate that the differences in diversity are not due to differences in sequencing depth, rarefaction analysis investigates alpha diversity (diversity within a sample) of randomly selected samples and different matrices can be used such as observed OTUs, Chao 1, Shannon index or Simpson's diversity (using `alpha_diversity.py`). The results from these analysis showed that all the diversity was captured from the sequencing depth utilised, so this demonstrated that all samples approached saturation or had plateaued using all metrics investigated. As all graphs using the different matrices showed saturation, only observed OTUs was represented here. (Figure 5. 2).

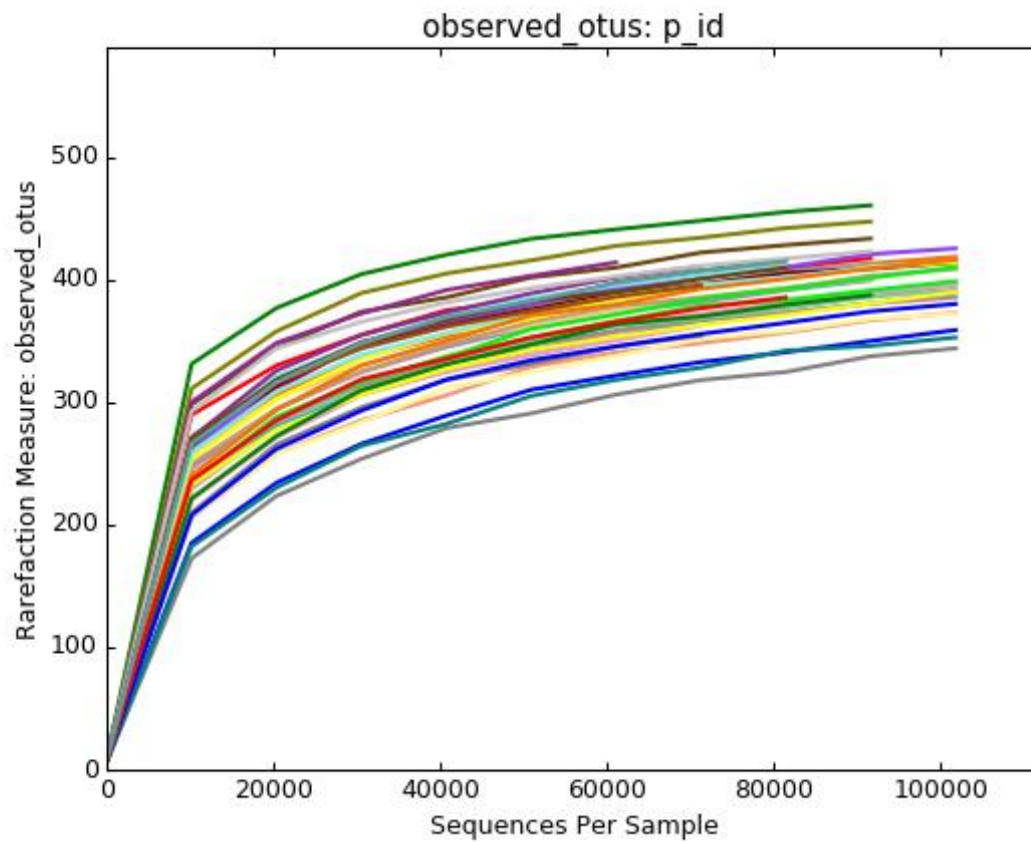


Figure 5. 2 Rarefaction analysis of samples tested according to OTUs and samples. Methods described in section 2.5.

### 5.2.2. Patient background

An initial cross-sectional analysis was performed on all patients included in this project (n=160) to investigate which clinical characteristics differentiate clusters of the oral microbiome. All RA patients (n=123) were classified according to their periodontal status: 23 periodontally healthy patients (15% of total RA diagnosed) and 100 patients with different degrees of periodontitis, from mild (n=49) to moderate (n=21) and severe (n=30).

From all patients in the systemically healthy group (n=37), 19 patients were classified as periodontally healthy (NoRA noPD), 17 patients were diagnosed with moderate to severe periodontitis (NoRAPD) and one patient had mild periodontitis. Clinical characteristics of the study population are represented in Table 5. 2.

Table 5. 2 Clinical characteristics of study population (n=160) according to periodontal and systemic condition. RA data not available in systemically healthy controls (-). Abbreviations: RA, rheumatoid arthritis; PD, periodontitis; CumPPD, cumulative probing depth; PPD, probing pocket depth; BoP, bleeding on probing; CAL, clinical attachment level; DAS, disease activity score; ESR, erythrocyte sedimentation rate; VAS, visual analogue score; IQR, inter-quartile range; n, number of patients per group. Methods described in section 2.5.

	RAPD (n=100)	RAnoPD (n=22)	noRAPD (n=18)	NoRA NoPD (n=19)
Female proportion	72%	78%	55.6%	63%
Ethnicity proportion				
-Asian	15%	4%	5.6%	11%
-White	53%	91%	78%	89%
-Black	6%	0%	11%	0%
Smoking proportion				
-Current	21%	13%	5.6%	5%
-Former	30%	21%	55.6%	11%
-Never	49%	61%	33.3%	84%
Alcohol consumption				
-Never	38%	13%	11%	15%
-1-4 times/month	37%	47%	16%	65%
-1-4 times/week	19%	26%	44%	15%
- >4 times/week	3%	13%	16.7%	5%
Age: median (IQR)	59 (18)	60 (14)	49 (9)	36 (16)
CumPPD: median (IQR)	86 (45)	12 (12)	111 (31)	10 (4)
PPD: median (IQR)	2.83(0.86)	2.34(0.34)	3.17(0.96)	1.5(0.29)
BoP: median (IQR)	0.19(0.29)	0.5(0.19)	0.38(0.24)	0.02(0.1)
CAL: median (IQR)	3.2(1.2)	2.4(0.64)	3.9(1.9)	1.7(0.24)
DAS28: median (IQR)	4.28(2)	3.42(2)	-	-
ESR: median (IQR)	14(24)	8(23)	-	-
VAS: median (IQR)	55(40)	41(24)	-	-
Tender joints: median (IQR)	7(13)	3.5(5)	-	-
Swollen joints: median (IQR)	1(2)	0(1)	-	-

### 5.2.3. Beta diversity analysis

Beta diversity studies the differences in bacterial communities between groups. This can be measured by simply studying the bacterial taxa that overlap (Bray Curtis dissimilarity matrix) or by using the phylogenetic distribution of species in each group (Unifrac distances) (Morgan and Huttenhower 2012).

To investigate which of the clinical characteristics studied create clustering on the oral microbiome, OTU data was plotted using Principal Coordinate analysis with different distance matrices (weighted and unweighted unifrac, Bray Curtis). The different clinical parameters, such as CAL, BoP, PPD, cumPPD, age, gender and smoking, were analyzed using the ANOSIM method (compare\_categories.py) which tests the strength and statistical significance of the sample groupings (Table 5. 3). From all the groupings, the only parameters that significantly shifted the oral microbiome were the systemic condition of RA (ANOSIM p value <0.001), periodontal classification (ANOSIM p value <0.001), cumulative PPD higher or lower than 40mm (ANOSIM p value =0.001), and CAL (ANOSIM p value =0.005) in both weighted and unweighted Unifrac distances. As both distances showed the results, Unweighted unifrac distances are represented as they illustrate better clustering (Figure 5. 3, Figure 5. 4, Figure 5. 5). In the 160 patients investigated here, there was no evidence that age, gender or smoking status significantly changed the oral microbiome.

ACPA status from 60 patients was available and only 13 patients were ACPA negative. For this reason, 15 ACPA positive patients, matched for gender, age and DAS28 severity, were selected. No significant differences in beta diversity between ACPA negative and positive patients were found.

Therefore, only RA and PD appeared to drive changes in the oral microbiome from all clinical characteristics included,



Table 5. 3 Grouping parameters tested for significance in clustering the oral microbiome. Test=ANOSIM of weighted unifrac distances (unweighted giving same results), significance if  $p < 0.05$ . Abbreviations: BoP, bleeding on probing; CAL, clinical attachment level; RA, rheumatoid arthritis; PPD, pocket probing depth. Methods described in section 2.5.

Grouping factor	Groups	ANOSIM significance
<b>Periodontal classification</b>	Eke&page 2012	$p=0.001$
<b>BoP</b>	Mean <10% vs 10-40% vs >40%	$p=0.02$
<b>CAL</b>	Mean <3mm, 3-4mm, >4mm	$p=0.005$
<b>RA</b>	RA vs systemically healthy	$p=0.001$
<b>PPD</b>	Mean 1-3 mm vs 3-4 mm vs >4 mm	Not significant
<b>Age</b>	20-40years old vs 40-60 vs >60 years old	Not significant
<b>Gender</b>	Male vs female	Not significant
<b>Smoking status</b>	Never vs current vs former	Not significant

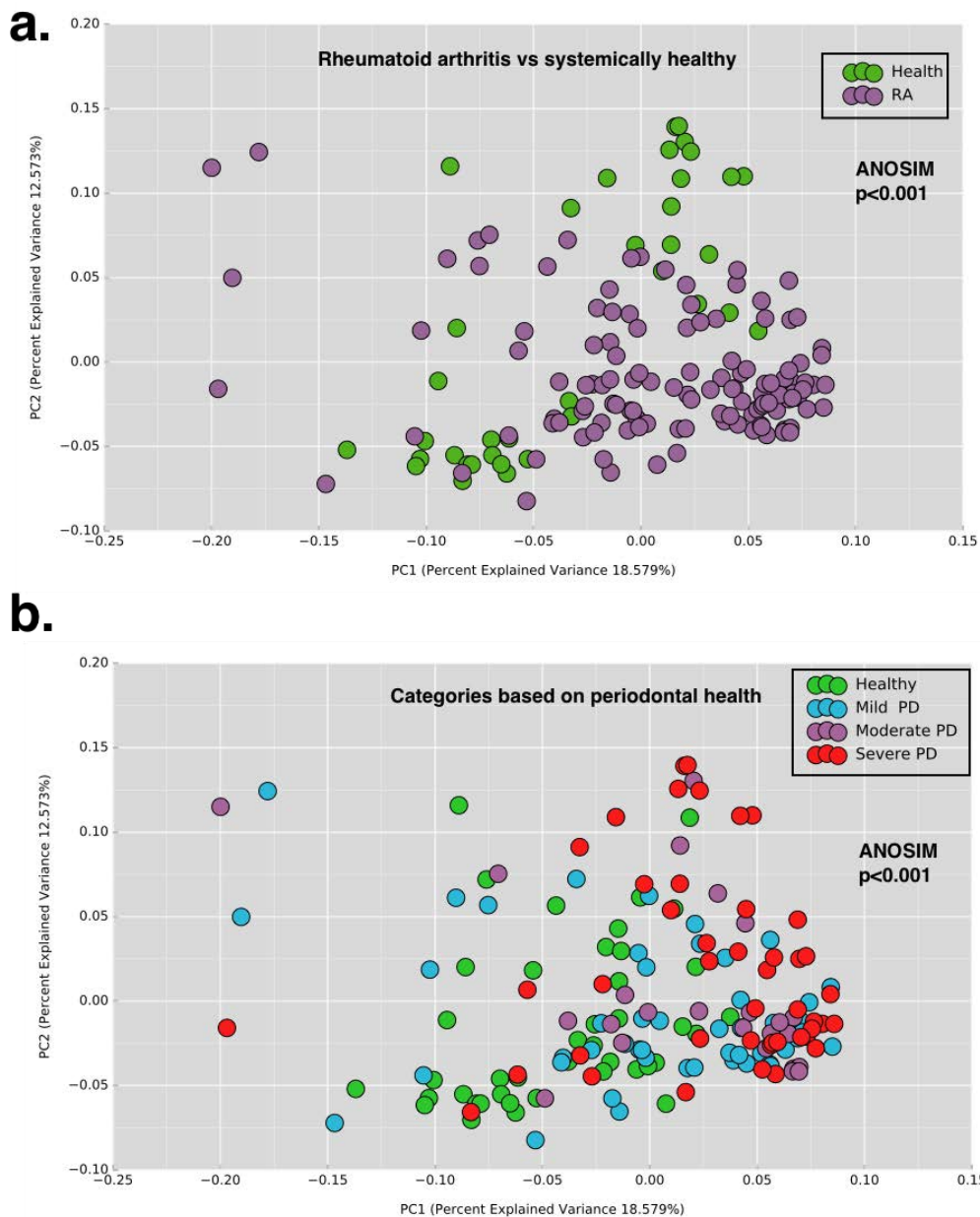


Figure 5. 3 Beta diversity of oral microbiome represented by principal coordinate analysis of unweighted unifracs distances according to significant categorisations (n=160). Abbreviations: RA, rheumatoid arthritis; PD, periodontitis. Significant clustering analysed with ANOSIM test (significance  $p < 0.05$ ). Methods described in section 2.5.

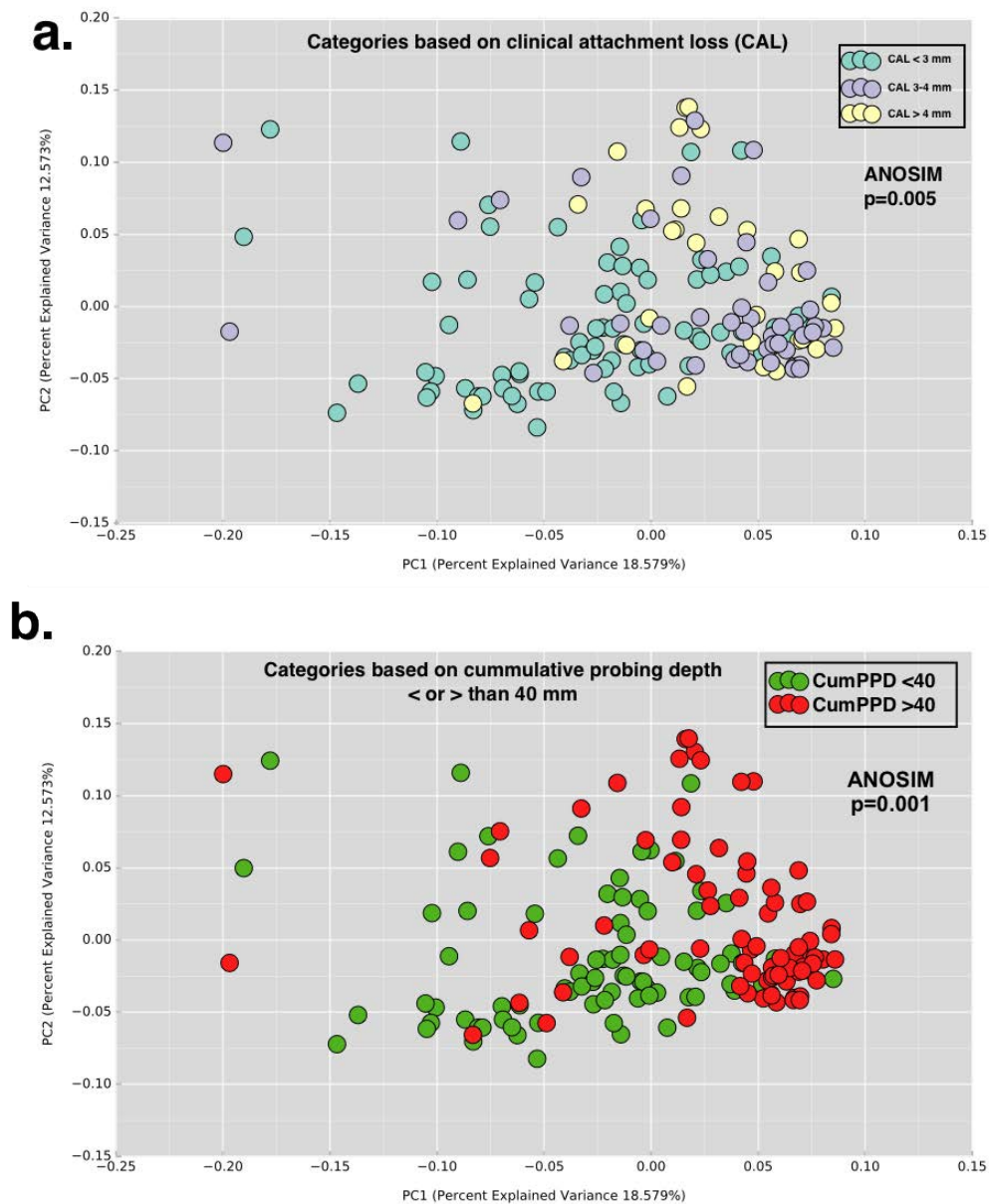


Figure 5. 4 Beta diversity of oral microbiome represented by principal coordinate analysis of unweighted unifracs distances according to significant periodontal categorisations (n=160). Significant clustering analysed with ANOSIM test (significance  $p < 0.05$ ). Methods described in section 2.5.

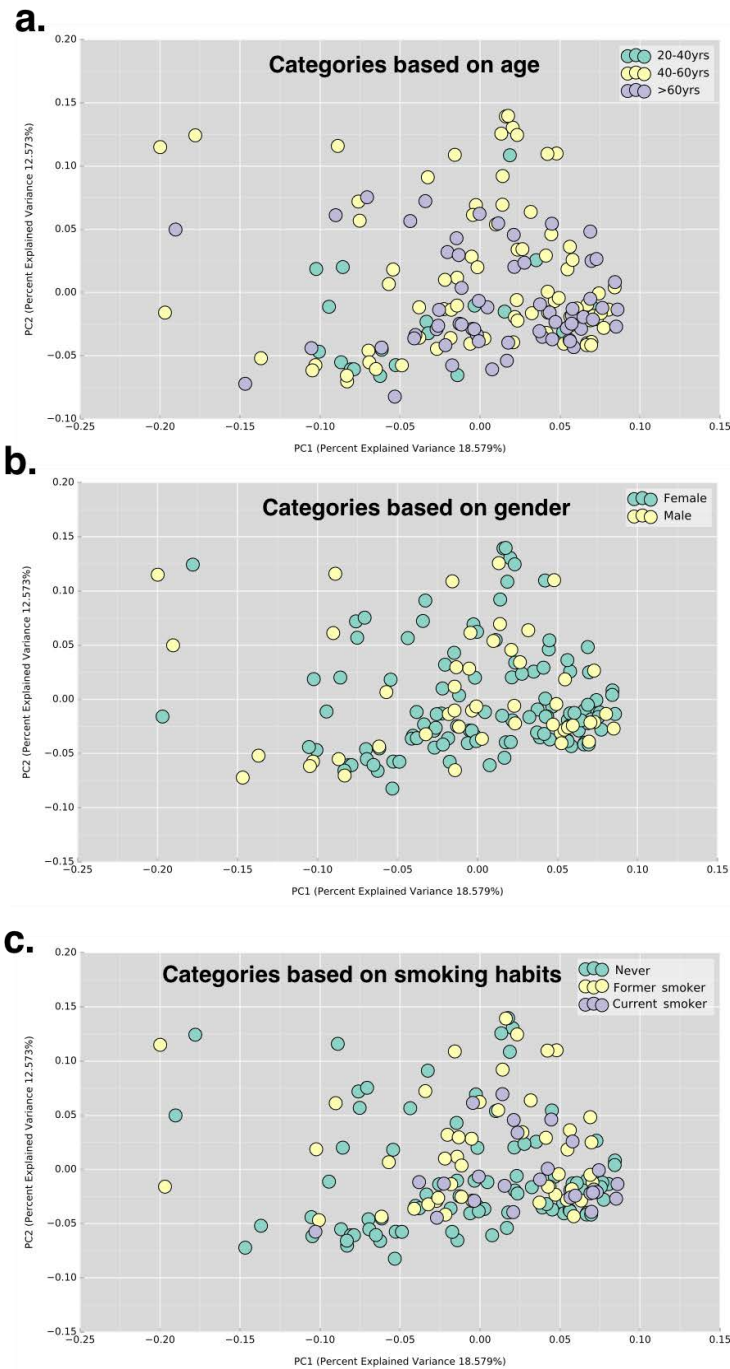


Figure 5. 5 Non significant parameters for categorization of oral microbiome. Principal Coordinates Analysis (PCoA) plots of unweighted unifracs distances according to age categorization (a.), gender (b.) and smoking habits (c.). Significant clustering analysed with ANOSIM test (significance  $p < 0.05$ ). Methods described in section 2.5.

### 5.3. RA and PD beta diversity analysis

Once evidenced that RA and PD were the main factors for changing the oral microbiome in the study population, all patients were classified according to their periodontal and RA status, resulting in 4 groups: 19 patients noRA noPD, 18 patients noRAPD; 23 patients RAnoPD; 100 patients RAPD. Various degrees of periodontitis were found in the latter RAPD group but, as 49 mild periodontitis/gingivitis patients did not belong to either periodontitis or health, those samples were removed from this cross-sectional analysis, leaving a total of 51 RAPD (moderate and severe periodontitis) samples remaining for further analysis.

Firstly, a beta diversity analysis was performed between the four groups using principal coordinate analyses of unifrac distances (Figure 5. 6). Beta diversity analyses measures the whole community between groups and there are different approaches to measure the distances between the species in each sample. Unifrac distances use the phylogenetic tree to measure the distances between the species depending on the evolutionary branches. While unweighted unifrac just accounts for presence and absence of lineages (community membership), weighted unifrac takes into account the relative abundances of the species, so that the branches are weighted depending on how much species there are in each branch. Weighted unifrac emphasises on the very abundant species), while unweighted unifrac also shows the not so abundant or rare species, so analysis

of both distances is useful to elucidate which species are contributing to the difference (Lozupone, Lladser et al. 2011).

Principal Coordinate Analysis for both weighted and unweighted unifrac showed significant differences in the communities for both approaches (ANOSIM  $p < 0.001$ ), demonstrating that the differences are not attributable to the rare biosphere only. Other distances can be used that do not account for phylogenetics but just look at the differences or similarities between species, such as bray Curtis, which also showed significant differences in this comparison ( $p < 0.001$ ).

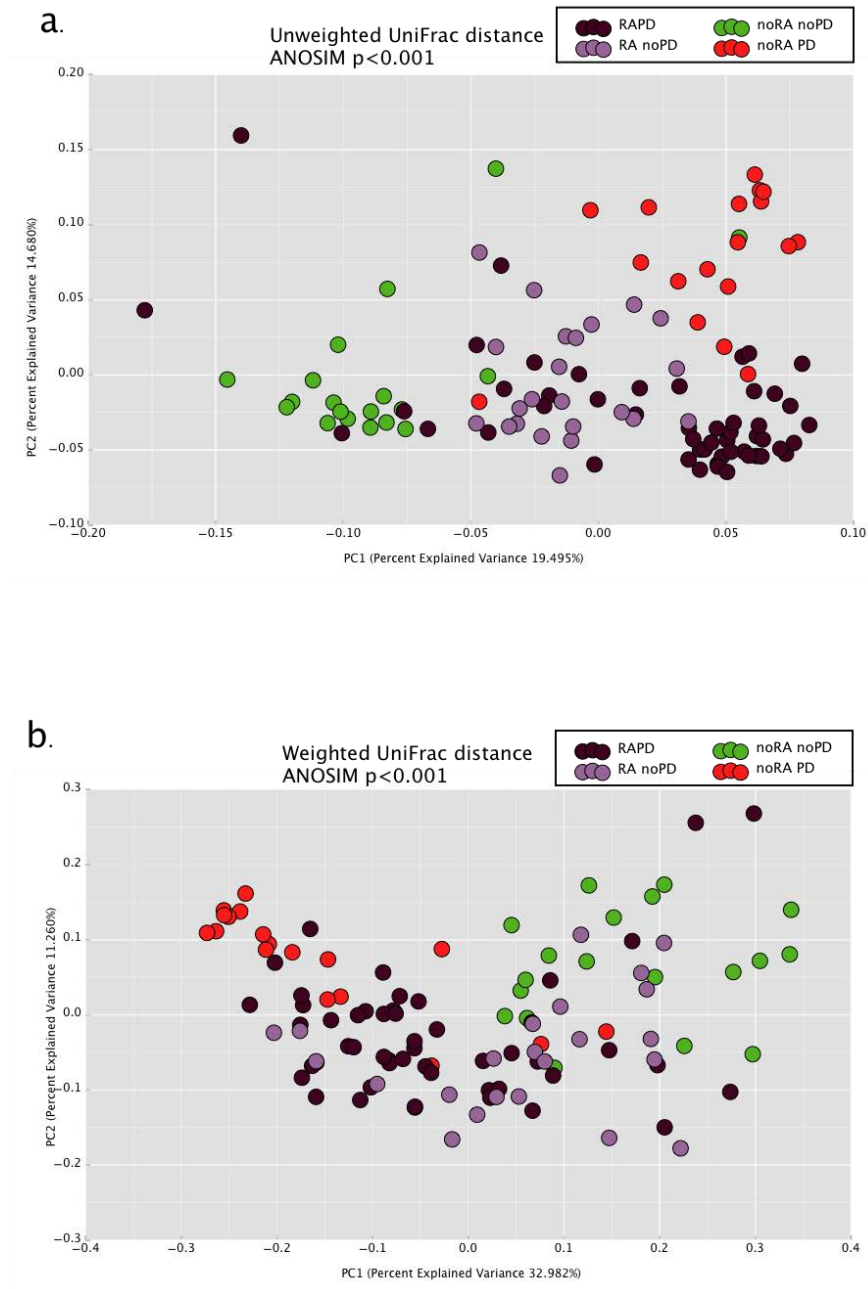


Figure 5. 6 Principal Coordinates Analysis (PCoA) plots of unweighted (a.) and weighted (b.) unifracs distances according to RA and periodontitis grouping. Significant clustering analysed with ANOSIM test (if  $p < 0.05$ ). Methods described in section 2.5.

#### 5.4. Effect of RA in periodontally healthy microbiome

At least two next-generation sequencing studies have investigated the RA microbiome (Zhang, Zhang et al. 2015) (Scher and Abramson 2011) showing differences in diversity and structure of the oral microbiome of RA compared to controls. However, due to the high prevalence of periodontitis in the RA population compared to the controls, these differences could be due to the periodontal factor and not to the systemic condition, as periodontitis was not controlled in previous studies.

To investigate the association between RA and the oral microbiota, the subgingival microbiome of periodontally healthy patients with Rheumatoid arthritis (RA) was compared to the appropriate systemically healthy controls. A total of 3,963,291 16S rDNA sequences were analyzed from 41 periodontally healthy volunteers. The mean number of sequences per sample was 107,115 (minimum 69,626 and maximum 18,293,000).

##### 5.4.1. Clinical data of study volunteers

The clinical characteristics of the 22 RA and systemically healthy 19 controls are represented in Table 5. 2. A total of 41 periodontally healthy patients were included in the study, composed by 19 systemically healthy controls and 22 RA.



No significant differences in smoking history, alcohol consumption, sex or ethnicity were found. Only mean age was found to be significantly higher in the RA group (mean age RA=60, mean age NoRA=36;  $p<0.01$  Mann-Whitney U test), and periodontal parameters such as CAL (mean RA=2.4, mean NoRA=0.7;  $p<0.01$  Mann-Whitney U test) and PPD (mean age RA=2.3, mean age NoRA=1.6;  $p<0.01$  Mann-Whitney U test). However, although these periodontal parameters were statistically significantly higher in the RA group, the difference was very small and clinically insignificant and all patients were periodontally healthy (i.e. no pockets of 4mm or more).

#### 5.4.2. Beta diversity

Principal Coordinate Analysis (PCoA) of both unweighted and weighted UniFrac distances demonstrated significant clustering of the microbiomes based on RA status (Figure 1a,  $p=0.001$ , ANOSIM test), indicating that these groups differed both in presence or absence of lineages (community membership), as well as in the relative abundances of lineages within communities (community structure).

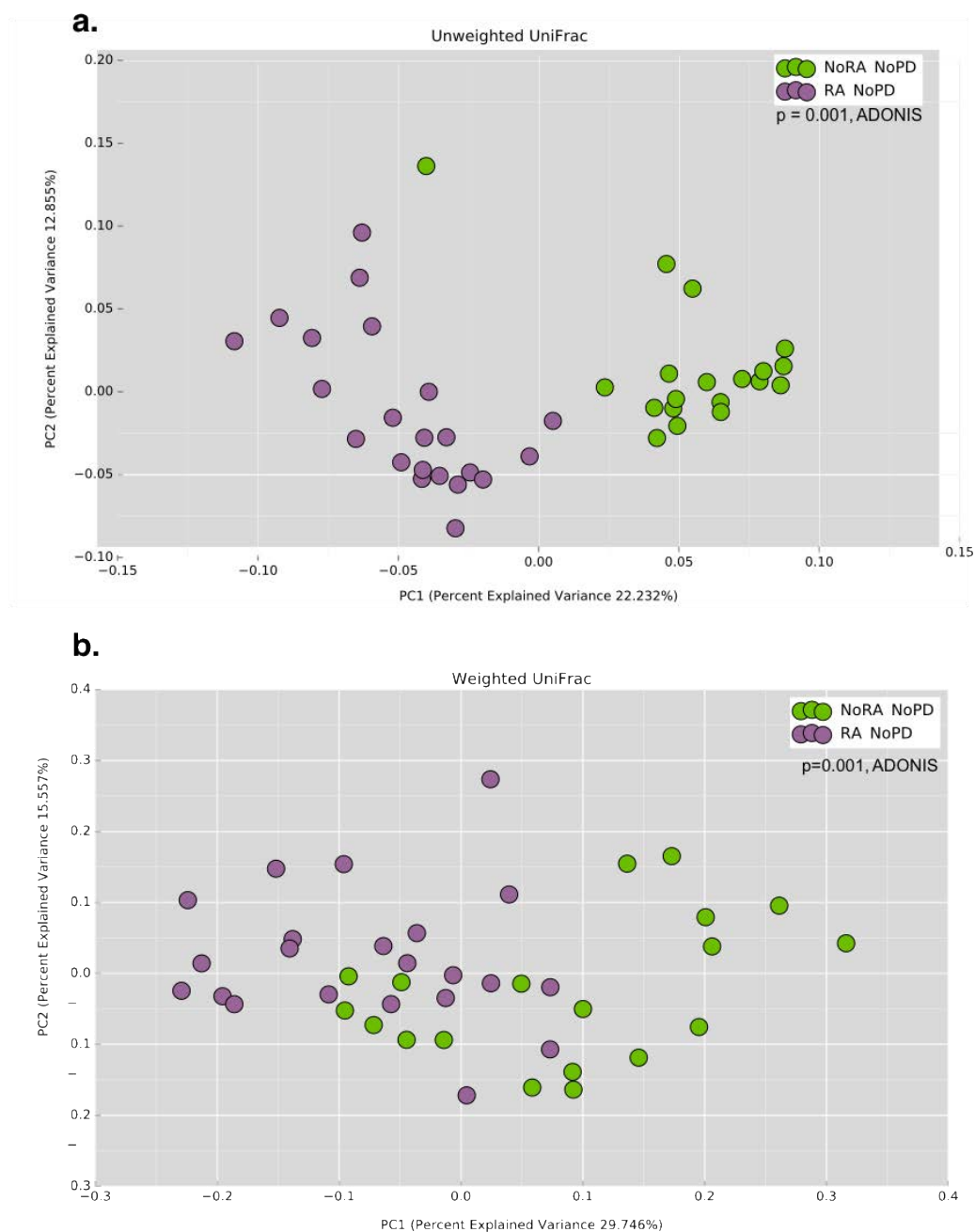


Figure 5. 7 Differences in beta diversity between periodontally healthy subjects with and without Rheumatoid Arthritis (RA). Principal Coordinates Analysis (PCoA) plots of unweighted (a.) and weighted (b.) of unifrac distances. n NoRA NoPD=19, n RA NoPD=22. Abbreviations: PD, chronic periodontitis. Methods described in section 2.5.

#### 5.4.3. Alpha diversity

Alpha diversity investigates the diversity of the species found in a sample or group, including the count of OTUs found (richness) and the distribution (evenness). Alpha diversity was investigated using Abundance Coverage Estimator (ACE) analysis, which showed no significant differences between the groups. However, there were differences in the distribution of the samples, as the no-RA group was homogeneously distributed (Levene test for homogeneity of variances  $p < 0.05$ ) while the RA group showed high variances between the groups (Figure 5. 8). When performing the Welsh-ANOVA test for different variances, a total of 171 OTUs were found to be significantly different distributed in abundance between the two groups (30.64% of OTUs).

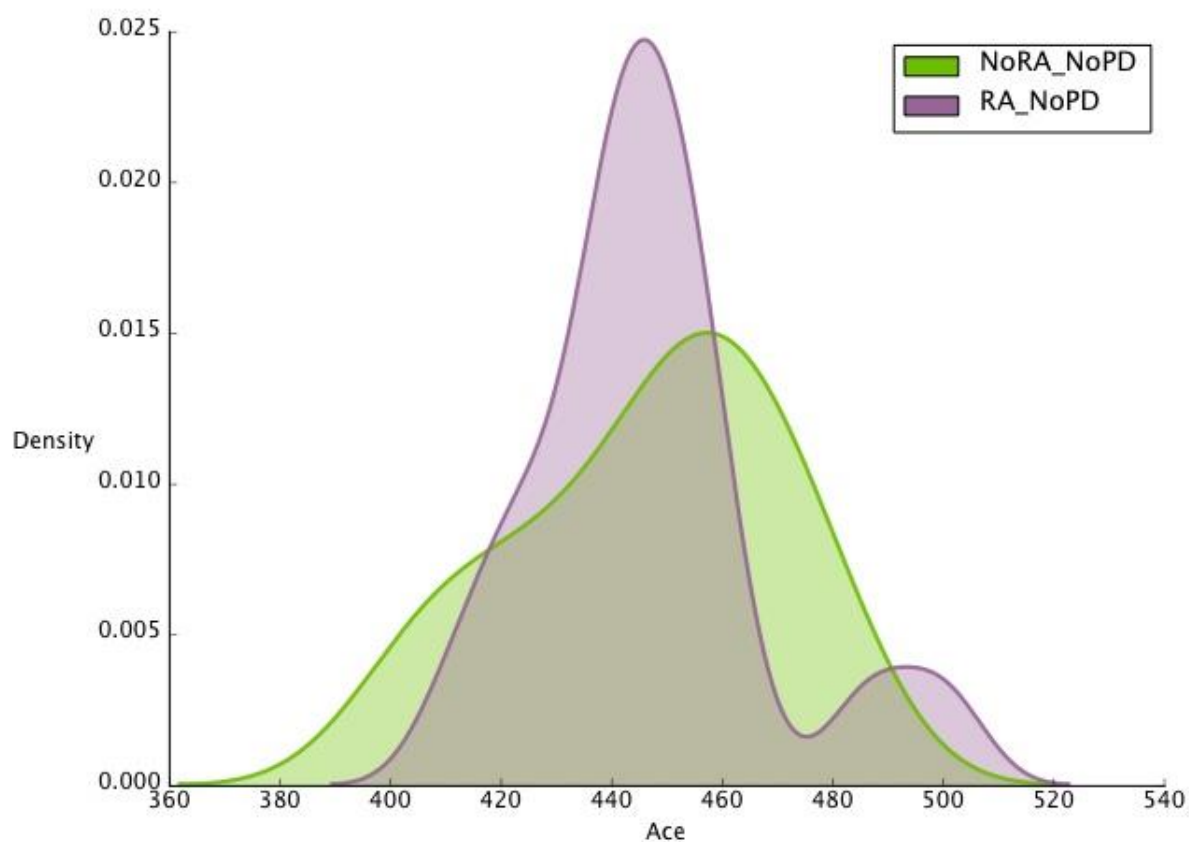


Figure 5. 8 Differences in alpha diversity between periodontally healthy subjects with and without Rheumatoid Arthritis (RA). Kernel plots of alpha diversity (Abundance-based Coverage Estimator (ACE). The peak indicates the median values for each group, and the x-axis shows the data range. n NoRA NoPD=19, n RA NoPD=22. Methods described in section 2.5.

#### 5.4.4. Gram and oxygen characteristics

Once established that the oral microbiome from RA patients differed from controls in both community membership and structure, group analysis was performed to investigate if these differences were reflected in the Gram staining characteristics and oxygen requirements of the two communities. In the gingival pocket, anaerobic bacteria (grows in the absence of oxygen) are typically related to disease, while facultative anaerobes (capable of replicating in aerobic and anaerobic conditions) are associated with health (Wade 2013).

The Gram staining characteristics and oxygen requirements of the two microbial communities are represented in Figure 5. 9, which is explored based on the taxonomic composition. Significant differences were found ( $p < 0.05$  Wilcoxon rank-sum test), showing that RA<sub>NoPD</sub> had more abundance of anaerobic species compared to NoRA<sub>NoPD</sub>, both Gram-negative anaerobes (46.7% in RA, 39.9% in No RA; Mann-Whitney U test  $p = 0.001$ ) and more Gram-positive facultative (24% in RA, 28% in No RA; Mann-Whitney U test  $p = 0.042$ )

The No RA patients presented a higher abundance of facultative species (43.49% in NoRA vs 35.27% in RA), especially Gram-negative aerobes (15.34% vs 11.29%; Mann-Whitney U test  $p = 0.007$ ). However, qualitatively (not considering abundance, just the bacteria found in each group) there were no differences in gram staining characteristics and oxygen requirement between groups, showing

that the determinant factor to differentiate the characteristics of the two communities was the abundance of the species.

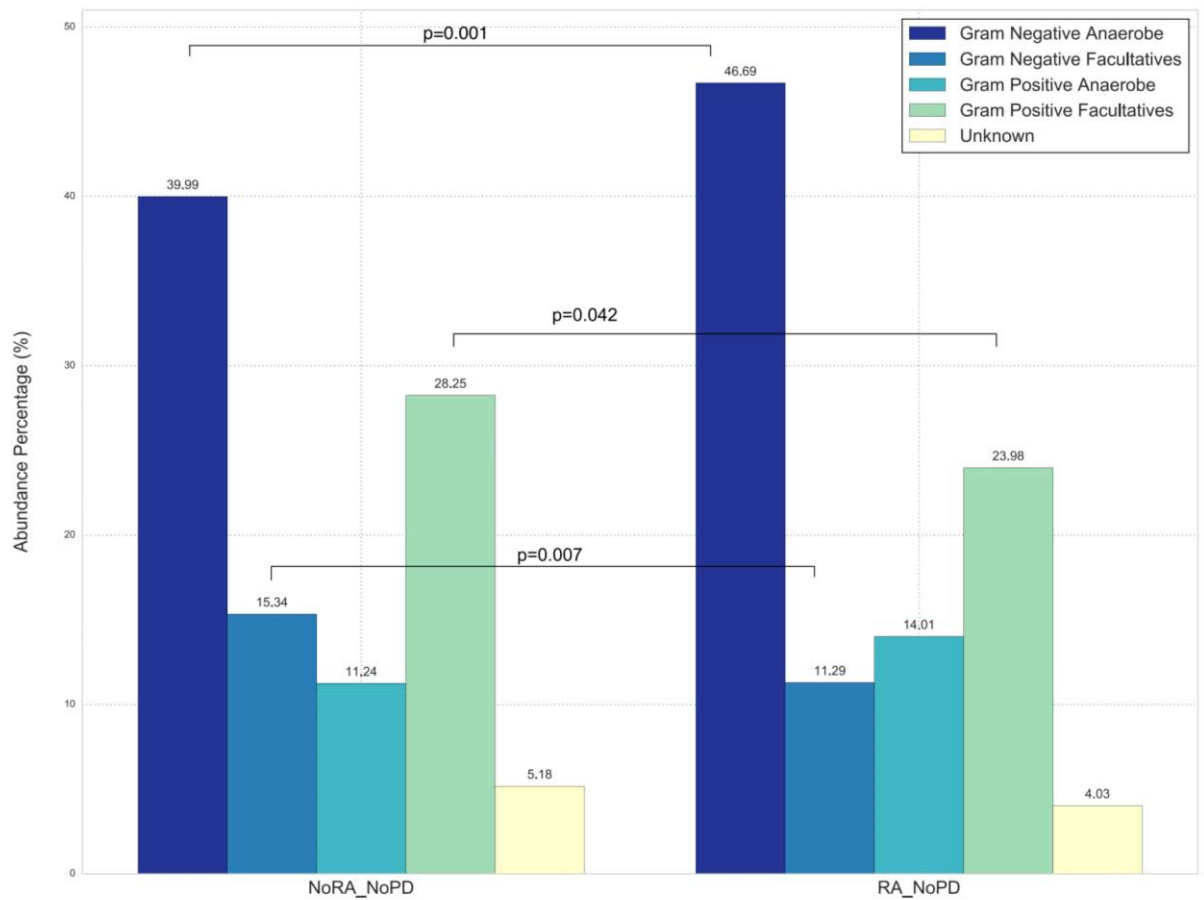


Figure 5. 9 Differences between RA and no RA in abundance of species by gram staining and oxygen requirement characteristics. Mann-Whitney U-test, significance if  $p<0.05$ . Abbreviations: RA, rheumatoid arthritis; PD, periodontitis. Methods described in section 2.5.

#### 5.4.5. Core microbiome

Due to the heterogeneity of the subgingival microbiome among individuals, the most common species (species found in  $\geq 80\%$  of subjects) were identified in each group, to compare stable associations between the RA microbiome and healthy controls. In the RA group, 364 OTUs were discovered as core microbiome and 27.7% of these (101 OTUs) differed significantly in relative abundance (community structure) with the control group and 38 species were unique in the RA group. In the No RA group, 326 core OTUs were identified and 16% of the core No RA microbiome differed significantly in structure (54 OTUs) and 11.7% were unique for the NoRA (38 OTUs).

Recent investigations demonstrate that while substantial microbial heterogeneity exists among healthy individuals, a robust core microbiome is identifiable in individuals who smoke or are pregnant (Mason, Preshaw et al. 2015), (Paropkari, Leblebicioglu et al. 2016). These findings suggest that RA, similarly to smoking and pregnancy, imposes a habitat that promotes the growth of certain organisms and shifts the microbial community typically associated with disease.

#### 5.4.6. Differences in relative abundance and unique species

To identify which species contribute to the differences found between the two communities, DESeq2 analysis was used to identify differentially abundant OTUs, with p-values  $< 0.05$  adjusted for multiple testing. The relative abundance



of 558 OTUs was identified as significantly different between the two groups, from 3,963,291 classifiable sequences. 41.9% of the community (229 OTUs) differed significantly in community structure and 19% (105 OTUs) in membership (absence/presence).

From the group of OTUs significantly more abundant in the RA group, the predominant species belonged to the genera *Actinomyces*, *Cryptobacterium* (the genera with the highest OR=36), *Dialister*, *Desulfovibrio*, *Fretibacterium*, *Leptotrichia*, *Prevotella*, *Selenomonas*, *Treponema*, and *Veillonellaceae* [G1]. In patients with RA, these significantly abundant species accounted for a median of 28% (range from 12-82%) of each individual's microbiome, indicating that these differences are not attributable to the rare biosphere.

In contrast, species from the genera *Aggregatibacter*, *Gemella*, *Granulicatella*, *Hemophilus*, *Neisseria* and *Streptococci* not only demonstrated lower abundances in RA but were also less frequently detected in these subjects.

#### 5.4.7. Network correlations

Bacterial presence in a biofilm is driven by inter-dependent nutritional and metabolic interactions and the study of these inter-species associations helps understand health and disease microbial communities (Duran-Pinedo, Paster et al. 2011). For this reason, Spearman's correlation analysis was performed on

the presence of core microbiome of each group and significant OTUs (with  $p < 0.05$  and  $r > 0.7$ ) were identified. Using Gephi (Bastian, Heymann et al. 2009), parse, congeneric networks were observed in controls, indicating that this is an ecological niche in equilibrium (Figure 5. 10, methods detailed in section 2.6). However, in the RA group, the network topology of RA patients revealed a highly connected grid with a robust intergeneric hub (Figure 5. 11). 83 of the 157 core species were incorporated in this hub with known pathogenic species belonging to *Treponema*, *Selenomonas*, *Filifactor*, *Campylobacter* and *Fretibacterium* tightly interwoven into this hub.

The identification of species with the highest modularity and degree of a network is interesting as it shows the OTUs that provides stability and robustness to the community (Duran-Pinedo, Paster et al. 2011). The modularity measurement indicates which OTUs are more densely connected and the degree shows the OTUs with the highest number of connections and the top 10 species were identified for each group (Figure 5. 10, Figure 5. 11). Therefore, network graph analysis was combined with DESeq and core microbiome analysis to examine co-occurrence patterns and identify important community members (network anchors). In the noRA noPD controls no network anchors were identified. In the RA group, 12 gram-negative species were identified as network anchors.

This data suggests that a small group of anaerobic bacteria play an important role controlling the flow of resources in the RA-influenced microbiome, implying that even small changes in these anchors could impact upon community

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assembly in people with RA. Interestingly, species traditionally associated with RA, for example, *P. gingivalis* and *A.actinomycescomitans*, were not part of the network cluster.

## Co-occurrence network noRA noPD

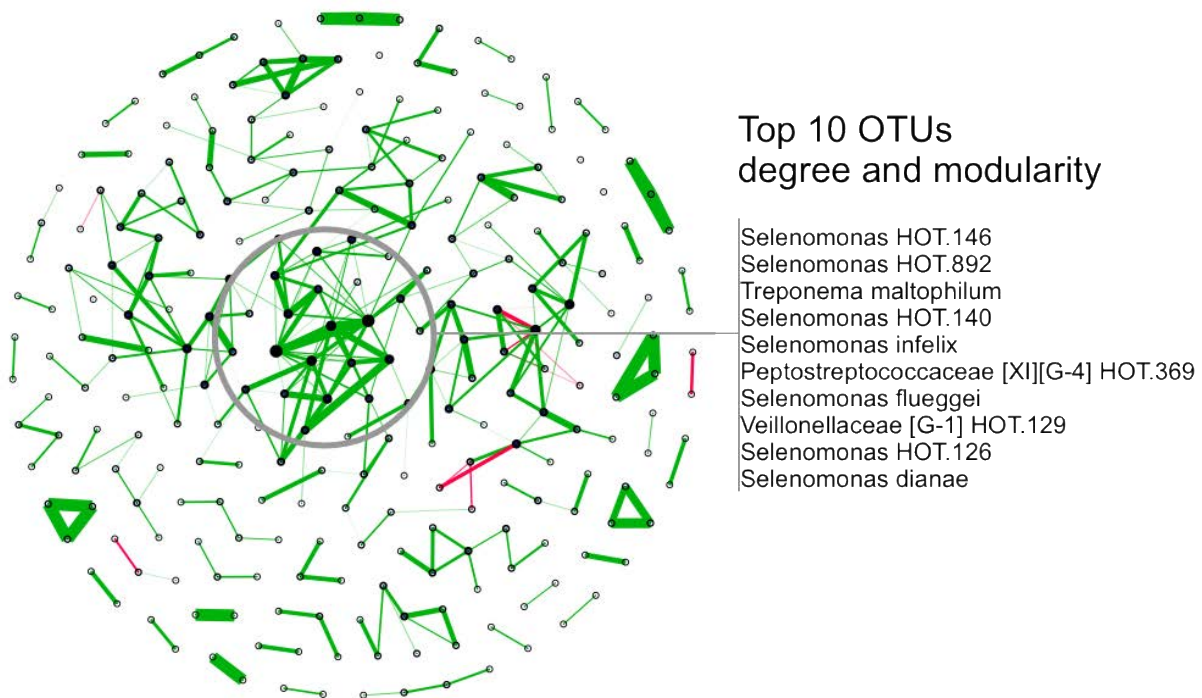


Figure 5. 10 Co-occurrence networks in periodontally and systemically healthy subjects (noRA noPD). Edges colored green for positive correlation and red for negative correlation and thickness represents the strength of the correlation. Nodes darkness and size represent the abundance of the species-level OTUs with significant and robust Spearman's correlation ( $p < 0.05$ ,  $\rho \geq 0.75$ ). Number of edges=324; number of nodes=263. Graph created with Gephi (Bastian, Heymann et al. 2009).

## Co-occurrence network RA noPD

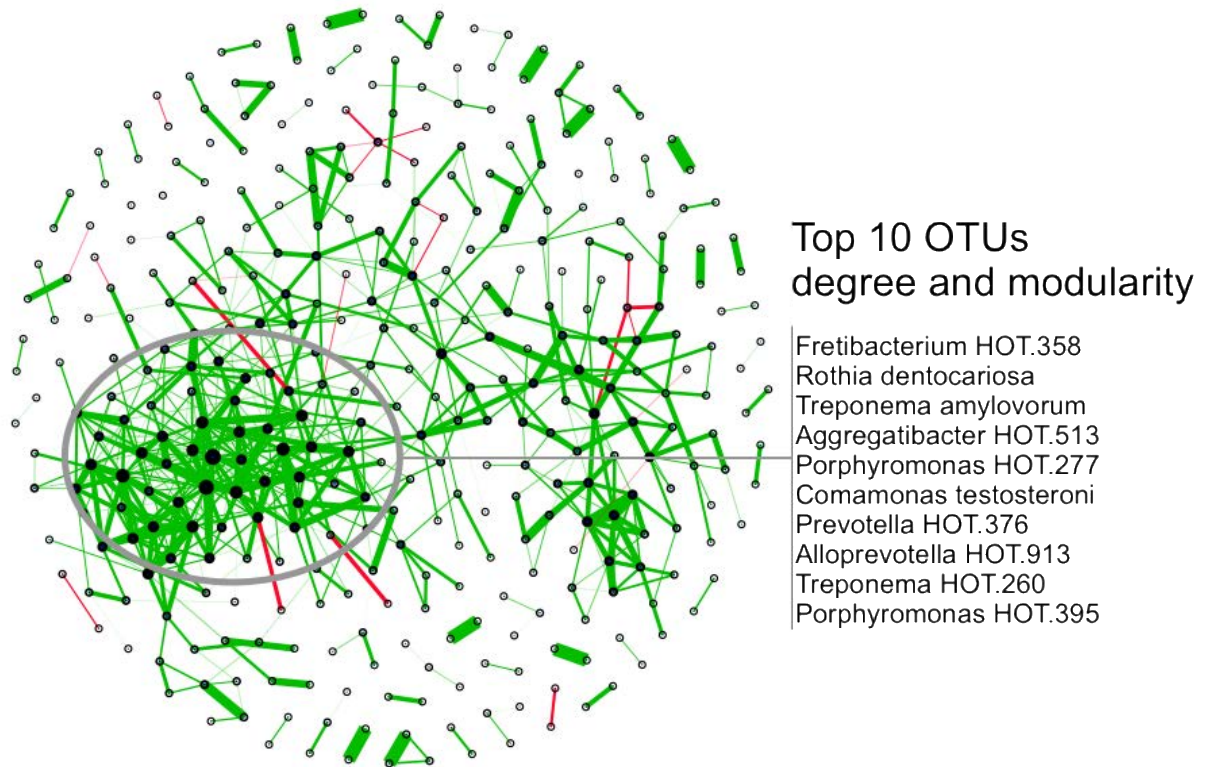


Figure 5. 11 Co-occurrence networks in periodontally healthy rheumatoid arthritis patients (RA noPD). Edges colored green for positive correlation and red for negative correlation and thickness represents the strength of the correlation. Nodes darkness and size represent the abundance of the species-level OTUs with significant and robust Spearman's correlation ( $p < 0.05$ ,  $\rho \geq 0.75$ ). Number of edges=561; number of nodes=227. Graph created with Gephi (Bastian, Heymann et al. 2009).

### 5.5. Effect of RA on the periodontitis microbiome

It has been shown that, RA patients, as well as having a higher prevalence of periodontitis, their periodontal disease is more severe than in the healthy population (27% of severe periodontitis in RA patients compared to 12% in non-RA) (de Smit, Westra et al. 2012). However, it is not known why in periodontitis patients, having RA is associated with a more severe periodontal phenotype. Studying the differences in the microbiome of periodontitis patients with and without RA could help elucidate why a more severe disease is associated with RA.

#### 5.5.1. Study volunteers

In the study of the differences in the community of the microbiome, similar sample sizes must be selected to avoid bias. As the RAPD group had 100 patients and the noRA PD group had 18 patients, the bias problem was solved by selecting 20 RAPD patients that were matched for age, gender and smoking status with the 18 noRA PD available patients. Periodontitis patients with mild disease were not included in this analysis as only moderate and severe patients were included. A total of 37 patients were selected and clinical parameters are described in Table 5. 4. No significant differences ( $p < 0.05$ ) were found when analysing differences in mean PPD, BoP and mean CAL between the groups (Mann Whitney-U test) but differences were found in age ( $p = 0.04$ ) with a mean difference of 5 years (standard error of difference = 0.15) older in the RA group (Table 5. 4).

Table 5. 4 Clinical characteristics of study population (n=37) according to systemic condition. RA data not available in systemically healthy controls (-). The percentage not shown for each proportion represents missing data. Abbreviations: RA, rheumatoid arthritis; PD, periodontitis; CumPPD, cumulative probing depth; PPD, probing pocket depth; BoP, bleeding on probing; CAL, clinical attachment level; DAS, disease activity score; ESR, erythrocyte sedimentation rate; VAS, visual analogue score; IQR, inter-quartile range; n, number of patients per group. Methods described in section 2.5.

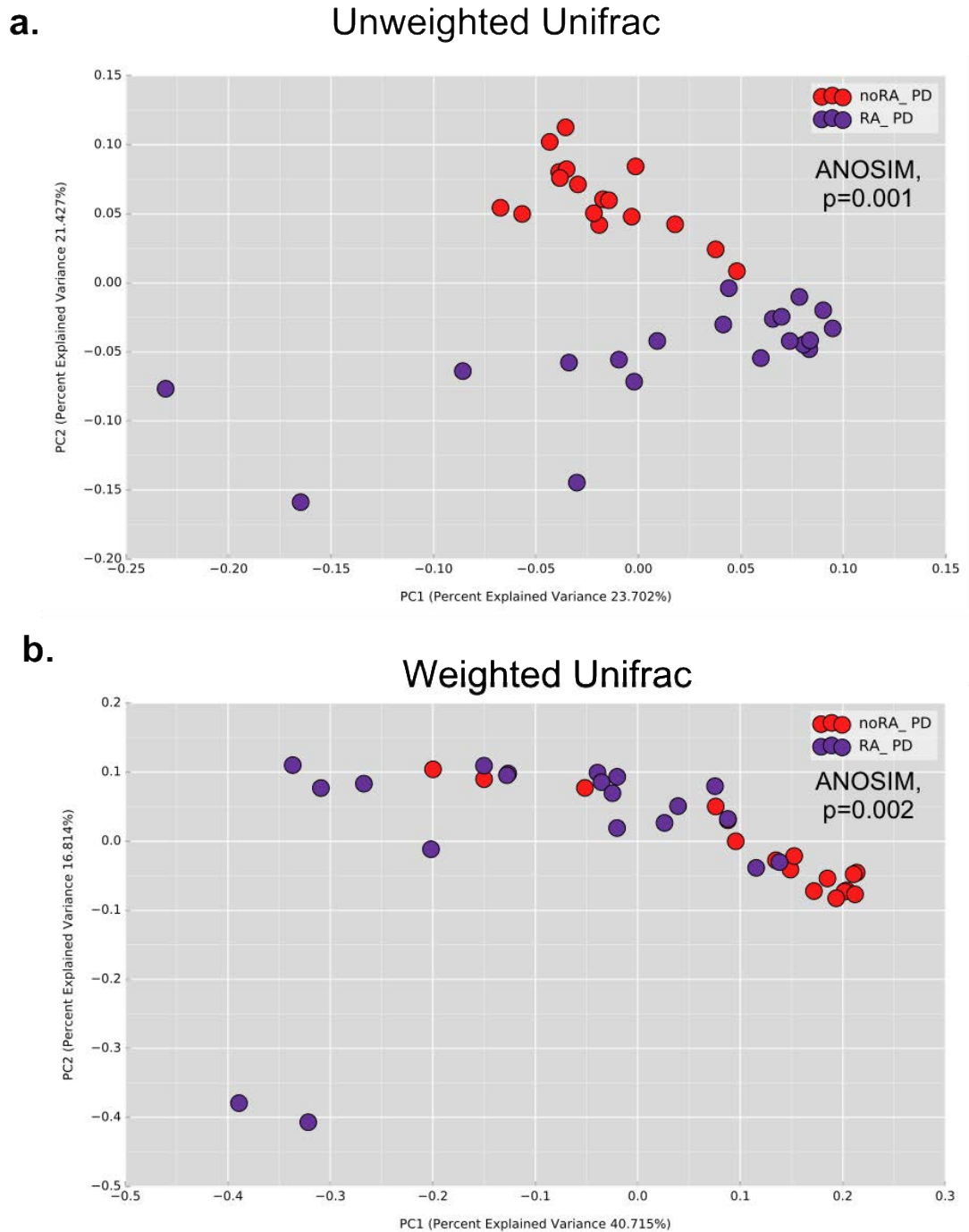
	RAPD (n=20)	noRAPD (n=18)
Female proportion	65%	55.6%
Ethnicity proportion		
-Asian	10%	5.6%
-White	55%	78%
-Black	10%	11%
Smoking proportion		
-Current	10%	5.6%
-Former	50%	55.6%
-Never	40%	33.3%
Alcohol consumption		
-Never	30%	11%
-1-4 times/month	35%	16%
-1-4 times/week	25%	44%
- >4 times/week	5%	16.7%
Age: median (IQR)	52 (13)	49(9)
CumPPD: median (IQR)	89(56)	111(24)
PPD: median (IQR)	3.47(1.6)	3.17(0.96)
BoP: median (IQR)	0.26(0.35)	0.38(0.24)
CAL: median (IQR)	3.65(1.9)	3.9(1.9)
DAS28: median (IQR)	4.43(2.27)	-
ESR: median (IQR)	14 (25)	-
VAS: median (IQR)	55 (50)	-
Tender joints: median (IQR)	7 (12)	-
Swollen joints: median (IQR)	1(2)	-

### 5.5.2. Beta diversity

Beta diversity analysis of the oral microbiome was performed to analyse differences between periodontitis patients with RA and without RA. From the 38 patients included, a total of 565 OTUs were identified.

Principal Coordinate analysis (PCoA) of both unweighted and weighted unifrac distances demonstrated significant clustering based on RA status using the ANOSIM test ( $p=0.001$ :  $R=0.24$  for weighted,  $R=0.5$  for unweighted), indicating that these two periodontitis groups differed in both community membership (presence or absence of lineages) as well as in the community structure (relative abundances of lineages within communities).





### 5.5.3. Alpha diversity

Since it was discovered that in periodontitis, the RA microbiome (RA<sub>no</sub>PD) differs from systemically healthy (NoRA<sub>no</sub>PD), comparison between alpha diversities was performed.

No significant difference was found between both alpha diversity curves (Mann-Whitney U test of ACE  $p=0.1$ ) (Figure 5. 13). However, similarly to section 5.3, the graphs showed different distribution, while noRA<sub>perio</sub> follows a skewed but symmetric distribution, RA<sub>perio</sub> had a higher diversity (0.8 to 8 compared to 3.5 to 7 range of Shannon Diversity) and two peaks of density, showing a more uneven distribution of the diversity in the RAPD group similarly to the RA<sub>no</sub>PD group.

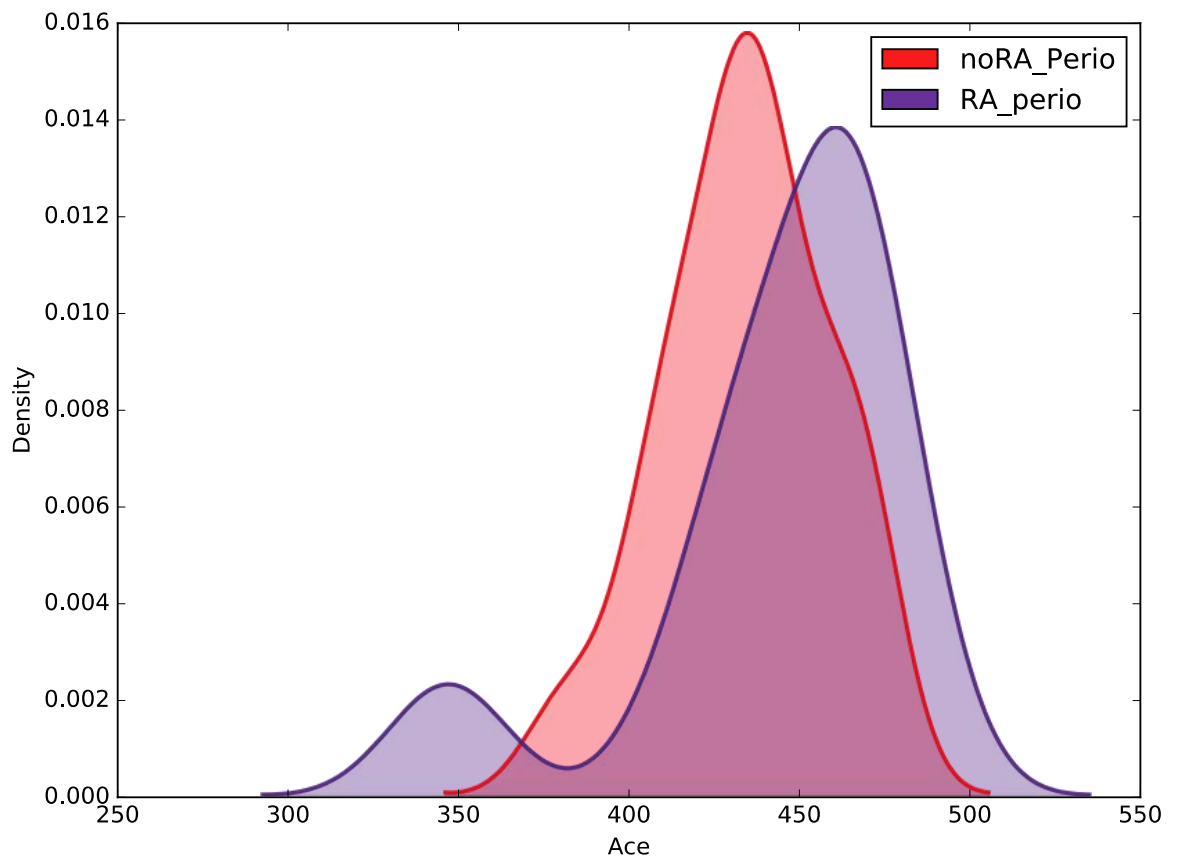


Figure 5. 13 Differences in alpha diversity between periodontitis patients with and without Rheumatoid Arthritis (RA). Kernel plots of alpha diversity (Abundance-based Coverage Estimator (ACE). The peak indicates the median values for each group, and the x-axis shows the data range. n RA\_perio=18, n noRA\_perio=20. Methods described in section 2.5.

#### 5.5.4. Gram and oxygen characteristics

The Gram staining characteristics and oxygen requirements of the periodontitis patients with and without RA are represented in Figure 5. 14. Following the opposite trend to the comparison between RA and noRA with healthy periodontium, systemically healthy periodontitis patients (RA noPD) presented a significantly higher proportion of obligate anaerobes (especially Gram negative, Mann-Whitney U test  $p=0.002$ ) and a lower proportion of Gram positive facultative (Mann- U test,  $p=0.003$ ).

This is interesting as it could show that, while in periodontal health, RA shifts the microbiome towards a more anaerobic environment, in periodontitis, the differences between RA and noRA are not due to an accumulated effect of anaerobic environment. In the case of patients with periodontitis and RA, it seems that the predominant bacteria are different.

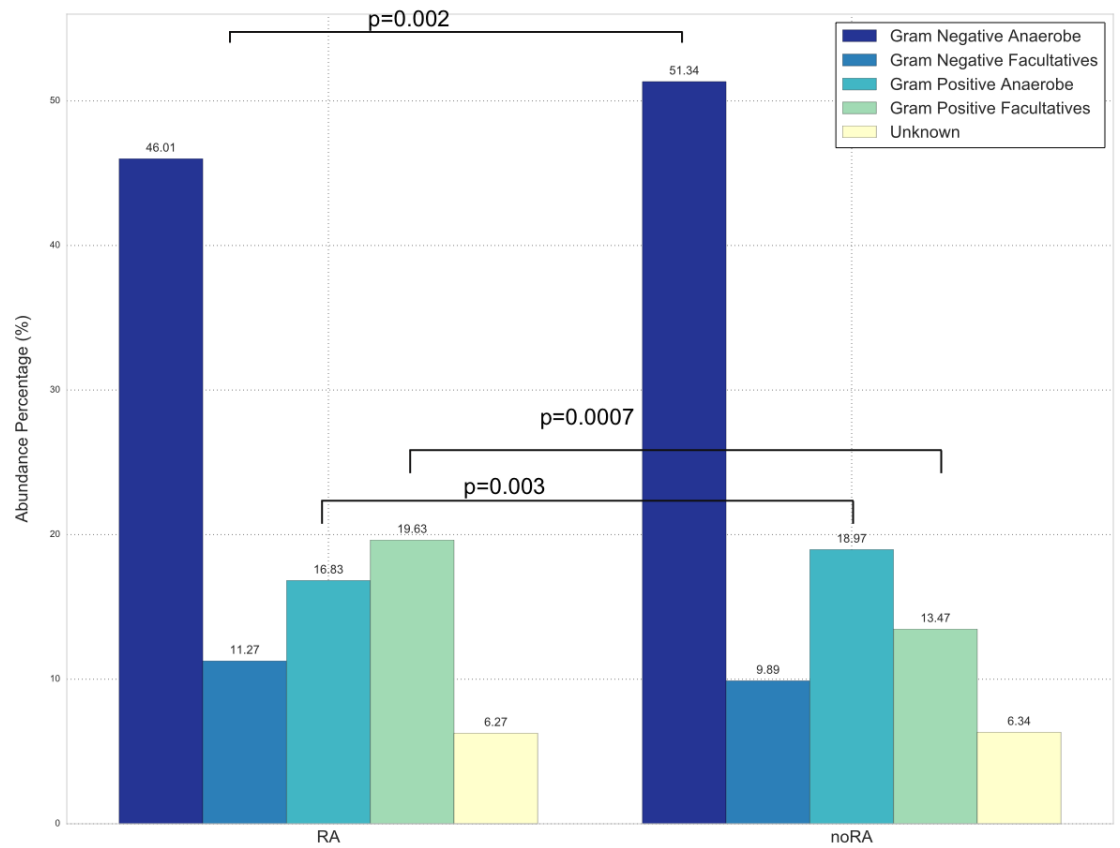


Figure 5. 14 Differences between RA and no RA patients with periodontitis in abundance of species by gram staining and oxygen requirement characteristics. Mann-Whitney U-test, significance if  $p < 0.05$ . Abbreviations: RA, rheumatoid arthritis; PD, periodontitis. Methods described in section 2.5.

#### 5.5.5. Core microbiome

The core microbiome of each group was defined as the microorganisms present in at least 80% of the patients in each group (Kumar, 2011). A total of 335 OTUs were discovered as core microbiome in the No RA (PD\_noRA) group and 313 OTUs as core microbiome in the RA group (RA\_PD). While these two core microbiomes had a majority of species in common, 56 (17%) of the RA core OTUs were not part of the noRA core, and 40 (13%) of no NoRA core microbiome was not part of the RA core.

The core community that was unique for RA was dominated by species belonging to *Capnocytophaga*, *Leptotrichia* and *Prevotella*. Interestingly, *P. gingivalis* was not part of the core microbiome of neither group, although these patients were diagnosed with moderate to severe periodontitis. While *A.actynomycetemcomitans* was part of the core microbiome in the systemically health periodontitis patients (noRA\_PD) but not the RAPD group, *C.curtum* was part of the core microbiome of RAPD but not the controls. This demonstrates that although the two groups had periodontitis, they differ in microbial community composition.

#### 5.5.6. Differences in relative abundance and unique species

Differential analysis (using DESeq2 from Qiime package) of the relative abundance of all OTUs were studied and 220 (23%) species were identified as

significantly different between the two groups, with p-values <0.05 adjusted for multiple testing, and Fisher's exact test to examine the frequency of detection. Only 7% of the species (37 OTUs) differed significantly in membership, 30 appearing uniquely in the RA group. *Cryptobacterium curtum* (OR=9.6, 95% CI 2.1-43.5) was again significantly more abundant in the RA group.

#### 5.5.7. Network correlations

Although similar network structures could be observed between the two groups, there were also differences. In the RAPD group, 250 core species were significantly and strongly correlated and 249 species were identified from the noRAPD. However, the systemically healthy group had more than 3 times higher number of correlations (354 correlations in the RAPD group, 1,195 correlations in noRAPD group), showing that in the systemically healthy group with periodontitis the species interconnected have a higher density of connections with each other and a more robust cluster is responsible for the disease.

In the RAPD group, species from the genera *Prevotella*, *Capnocytophaga*, *Treponema* and *Fusobacterium* were the species with the highest and strongest number of connections. 8 network anchors were found in the RAPD group which were species part of the core microbiome, significantly differently distributed compared to the noRAPD group and part of the top 20 species with highest modularity and degree in the network analysis. These species belonged to the genera *Treponema*, *Peptostreptococcus*, and *Lachnospiraceae*.

In the noRAPD group, 249 core species were significantly and strongly correlated. *P. gingivalis* was the most abundant species of the top 10 OTUs with highest modularity, which represents a species very densely connected with other OTUs and therefore a key OTU in the network of noRAPD microbiome. Interestingly, *A. actynomicetenscomintas* showed a low degree and low modularity.



## Co-occurrence network noRA PD

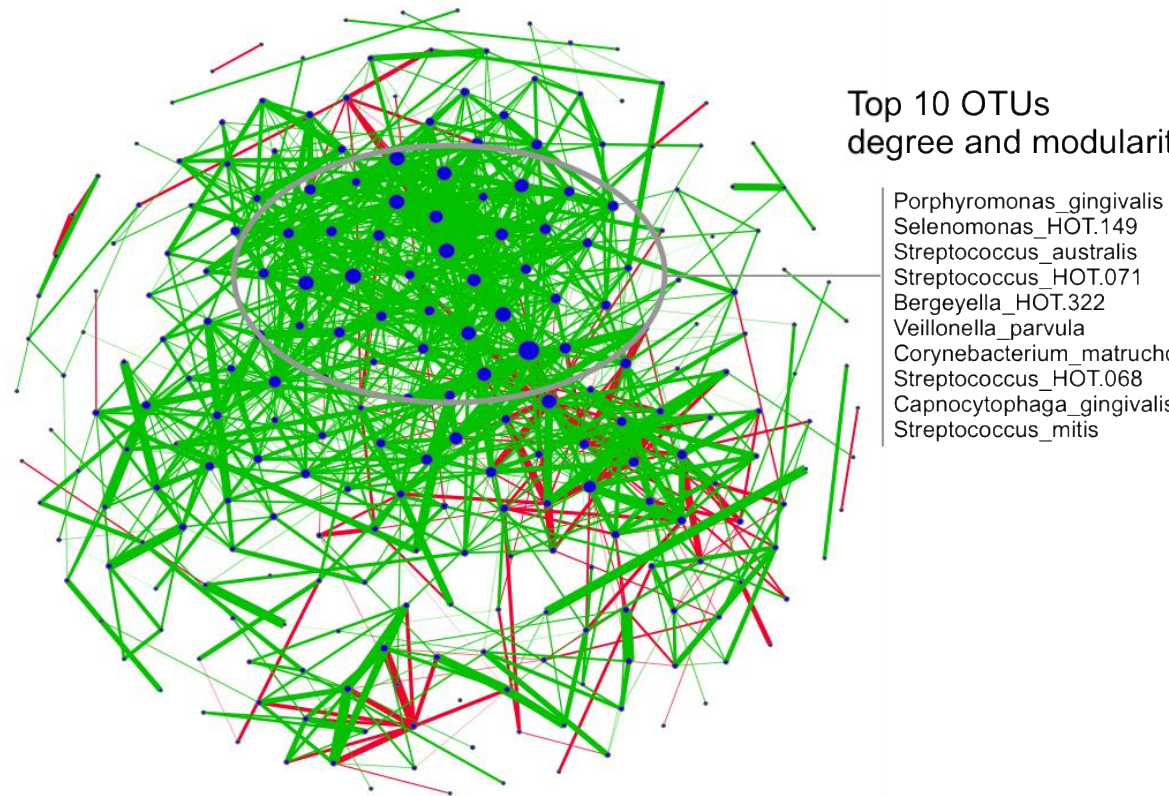


Figure 5. 15 Co-occurrence networks patients with periodontitis and systemically healthy (noRAPD). Edges colored green for positive correlation and red for negative correlation and thickness represents the strength of the correlation. Nodes darkness and size represent the abundance of the species-level OTUs with significant and robust Spearman's correlation ( $p < 0.05$ ,  $\rho \geq 0.75$ ). Number of edges=1183; number of nodes=249. Graph created with Gephi (Bastian, Heymann et al. 2009).

## Co-occurrence network RA PD

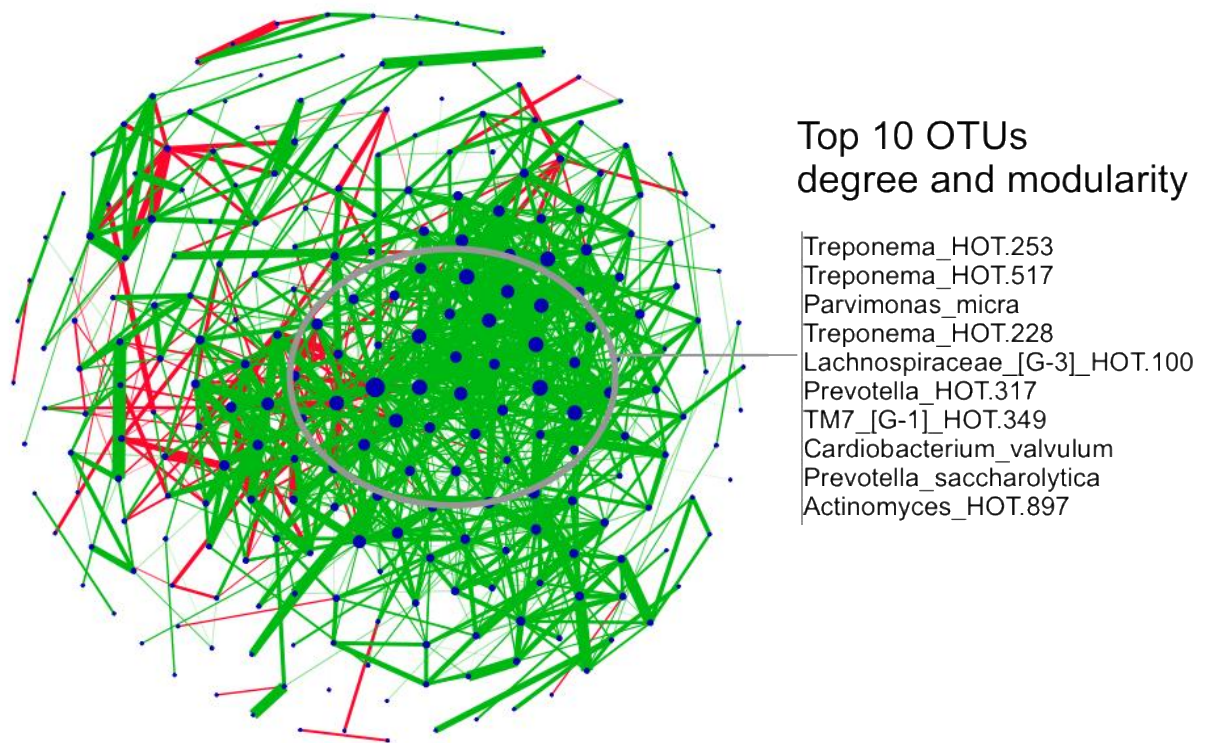


Figure 5. 16 Co-occurrence networks in rheumatoid arthritis patients with periodontitis (RAPD). Edges colored green for positive correlation and red for negative correlation and thickness represents the strength of the correlation. Nodes darkness and size represent the abundance of the species-level OTUs with significant and robust Spearman's correlation ( $p < 0.05$ ,  $\rho \geq 0.75$ ). Number of edges=1193; number of nodes=249. Graph created with Gephi (Bastian, Heymann et al. 2009).

## 5.6. Effect of PD in RA microbiome

### 5.6.1. Study volunteers

Clinical characteristics of periodontally healthy RA (RAnoPD) and all RA patients with periodontitis (RAPD) are described in Table 5. 2. To study the effect of periodontitis in the RA microbiome, all RA patients were studied and categorized depending on the periodontal status (Eke&Page classification). From the total RA patients (122), 22 were periodontally healthy, 49 had mild periodontitis, 21 had moderate periodontitis and 30 had severe periodontitis.

Due to the big differences in sample sizes between the groups, a subgroup of the RAPD mild/slight cohort was selected to equalize samples sizes, matching for age, smoking status and gender. The clinical characteristics of the study groups are represented in Table 5. 5, showing no differences in the demographics and a trend towards an increase of the RA disease markers with periodontal severity. This association between increased ESR or tender joints with periodontal severity, is not likely to be explained by other confounding factors such as smoking and age, as these factors did not differ between the groups.

Table 5. 5 Clinical characteristics of selected subjects with RA grouped by periodontal status. Abbreviations: RA, rheumatoid arthritis; PD, periodontitis; CumPPD, cumulative probing depth; PPD, probing pocket depth; BoP, bleeding on probing; CAL, clinical attachment level; DAS, disease activity score; ESR, erythrocyte sedimentation rate; VAS, visual analogue score; IQR, inter-quartile range; n, number of patients per group. Methods described in section 2.5.

	RA noPD (n=22)	RA mild PD (n=30)	RA moderate PD (n=21)	RA severe PD (n=30)
Female proportion	78%	77%	71%	60%
Ethnicity proportion				
-Asian	5%	10%	14%	13%
-White	92%	83%	70%	80%
-Black	3%	3%	15%	7%
Smoking proportion				
-Current	13%	20%	19%	23%
-Former	21%	43%	23%	33%
-Never	60%	27%	57%	44%
Alcohol consumption				
-Never	13%	26%	33%	47%
-1-4 times/month	47%	39%	27%	30%
-1-4 times/week	26%	27%	24%	17%
- >4 times/week	13%	4%	5%	4%
Age: median (IQR)	60 (17)	61 (20)	56 (17)	56 (12)
CumPPD: median (IQR)	12 (12)	35 (33)	69 (44)	71 (48)
PPD: median (IQR)	2.3 (0.3)	2.7 (0.38)	3.3 (0.5)	3.5 (1.5)
BoP: median (IQR)	0.06 (0.2)	0.14 (0.16)	0.33 (0.2)	0.26(0.4)
CAL: median (IQR)	2.5 (0.7)	2.9 (0.8)	3.3 (0.5)	4.03 (2.3)
DAS28: median (IQR)	3.4 (2.3)	4.2 (2.4)	3.6 (2.6)	4.8 (1.8)
ESR: median (IQR)	8 (23)	12 (23)	20 (28)	21 (0.7)
VAS: median (IQR)	45 (24)	55 (40)	50 (53)	60 (43)
Tender joints: median (IQR)	3.5 (5)	6.5 (14)	7 (13)	9 (13)
Swollen joints: median (IQR)	0 (1)	1 (4)	1 (3)	0 (2)

### 5.6.2. Beta and alpha diversity analysis according to different degrees of periodontitis

Analysis of the beta diversity of the oral microbiome of the 4 RA groups according to periodontal status was performed and significant differences were found (ANOSIM  $p = 0.02$ , Figure 5.17). To visualize the differences in beta diversity, the PCoA of different distance matrices were used, such as Bray Curtis, which measures the distances accounting for the evolutionary branches of the species and the relative abundance, or the bray curtis which assigns a value to the species in each sample and measures the differences. The ANOSIM results of both analysis were statistically significant ( $p = 0.02$ ), however, the R values were low (0.05), which showed that the differences inside groups were high.

Although due to the differences in distribution, differences in alpha diversity between the groups were not significant, the graphs of the periodontally healthy RA and mild periodontitis microbiome seemed to be similarly homogeneously distributed, while the RAPD moderate and severe were more heterogeneous (Figure 5.18). This showed that, periodontal status shapes the RA microbiome differentiating their beta diversity in community and membership and distribution of species with similarities between RA mildPD and RA noPD, and RA moderated and RA severe PD.

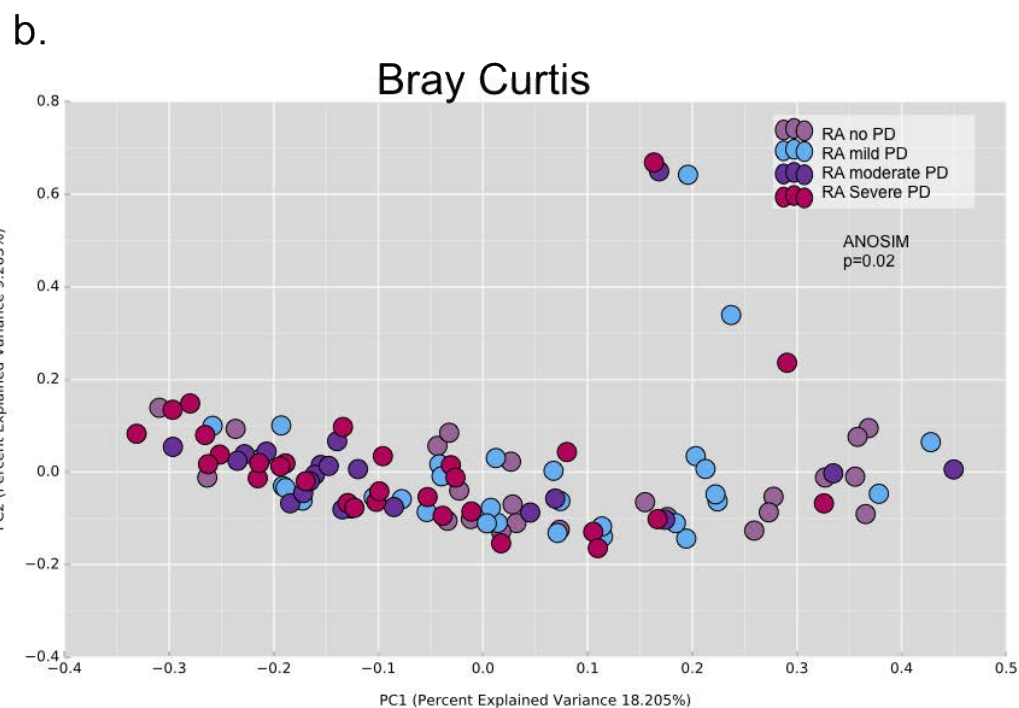
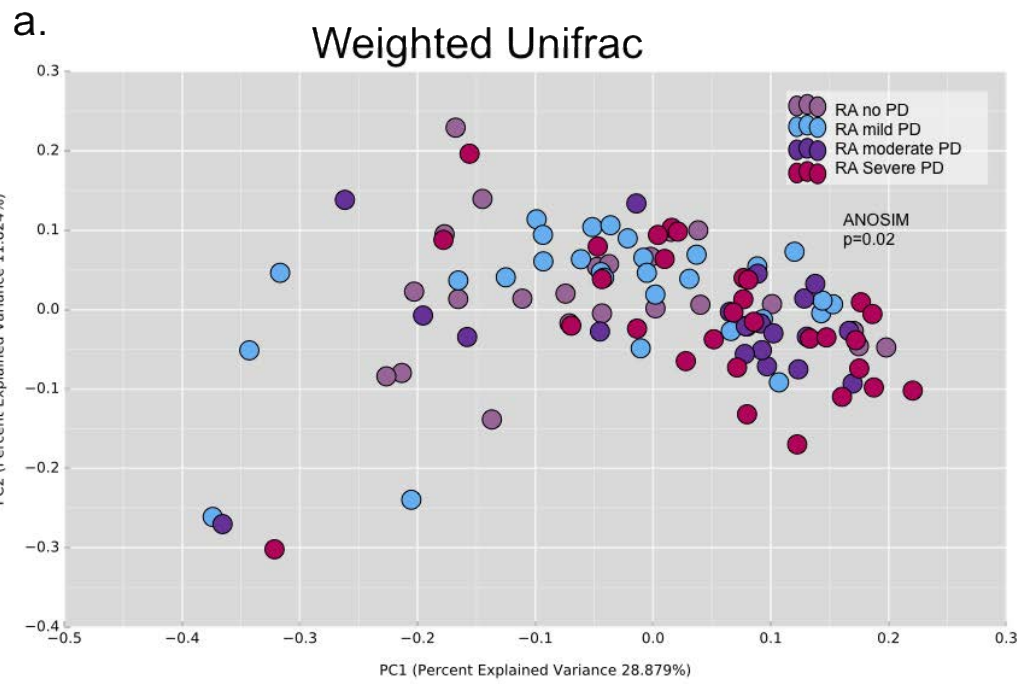


Figure 5.17 PCoA of weighted Unifrac (a) and Bray Curtis distances in RA patients grouped by periodontal status. n RA no PD=22; n RA mild PD=30, n RA moderate PD=21 ;n RA severe PD= 30. Test: ANOSIM, significance if  $p < 0.05$ . Abbreviations: RA, rheumatoid arthritis; PD, chronic periodontitis. Methods described in section 2.5.

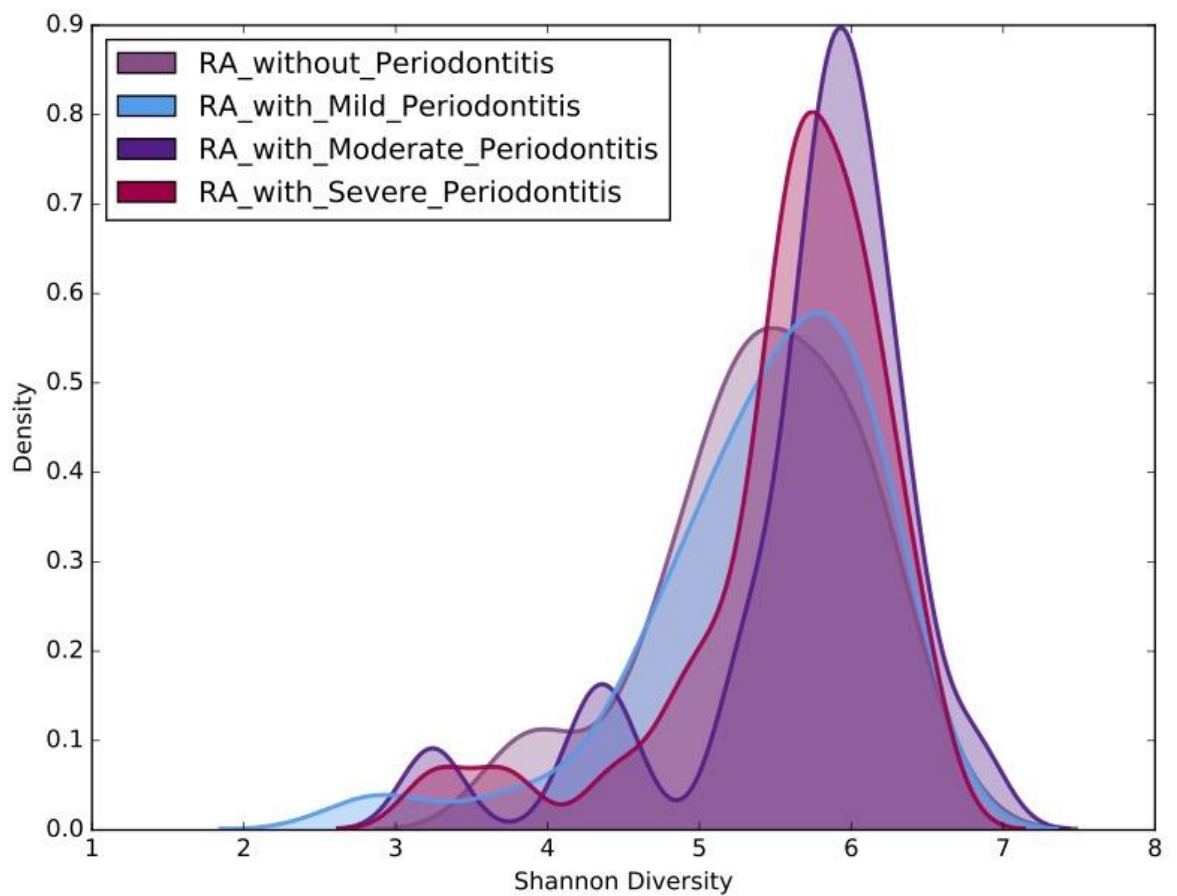


Figure 5. 18 Differences in alpha diversity between rheumatoid arthritis (RA) patients according to periodontal health. Kernel plots of alpha diversity (Shannon diversity analysis). The peak indicates the median values for each group, and the x-axis shows the data range. n RA no PD=22; n RA mild PD=30, n RA moderate PD=21 ;n RA severe PD= 30. Methods described in section 2.5

### 5.6.3. Beta diversity analysis of RAPD compared to RA noPD

Once it was clear that severity of periodontitis shaped both the RA clinical characteristics and the microbiome, a more in-depth analysis was performed to investigate what contributes to the differences between periodontal health and periodontitis (Figure 5.19), according to the definition of moderate and severe periodontitis (Eke&Page 2007). The RA no PD (n=22) and the RAPD (moderate and severe) groups were compared. To reach similar sample sizes, 26 RAPD patients were selected (moderate-severe, table 5.4), matching age, gender and smoking status. Significant differences were found in the oral microbiome of RA patients with and without periodontitis.



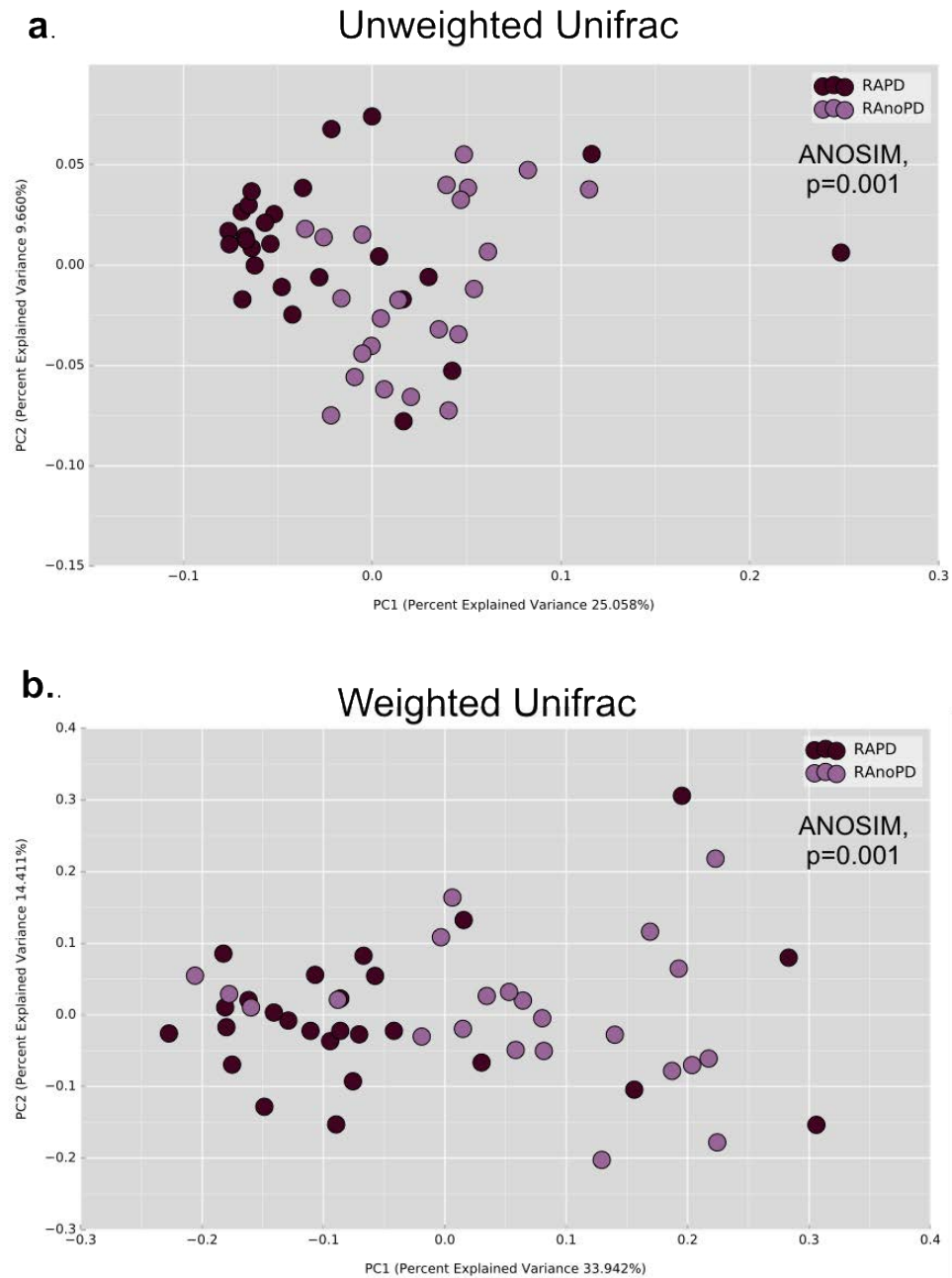


Figure 5. 19 Differences in beta diversity between rheumatoid arthritis patients (RA) with and without periodontitis (PD). Principal Coordinates Analysis (PCoA) plots of unweighted (a.) and weighted (b.) of unifrac distances. Test: ANOSIM, significance if  $p < 0.05$ . n RAnoPD=21; n RAPD=26. Methods described in section 2.5.

#### 5.6.4. Alpha diversity

The alpha diversity analysis shows significant differences (ACE and Shannon equitability Mann-Whitney U test  $p=0.05$ ) between the distribution of the two groups (Figure 5.20). The Shannon diversity measures the diversity within each group, accounting for richness and evenness. This analysis showed uneven distribution of both RA groups with a more skewed RAPD profile compared to a more homogeneous RAnoPD.

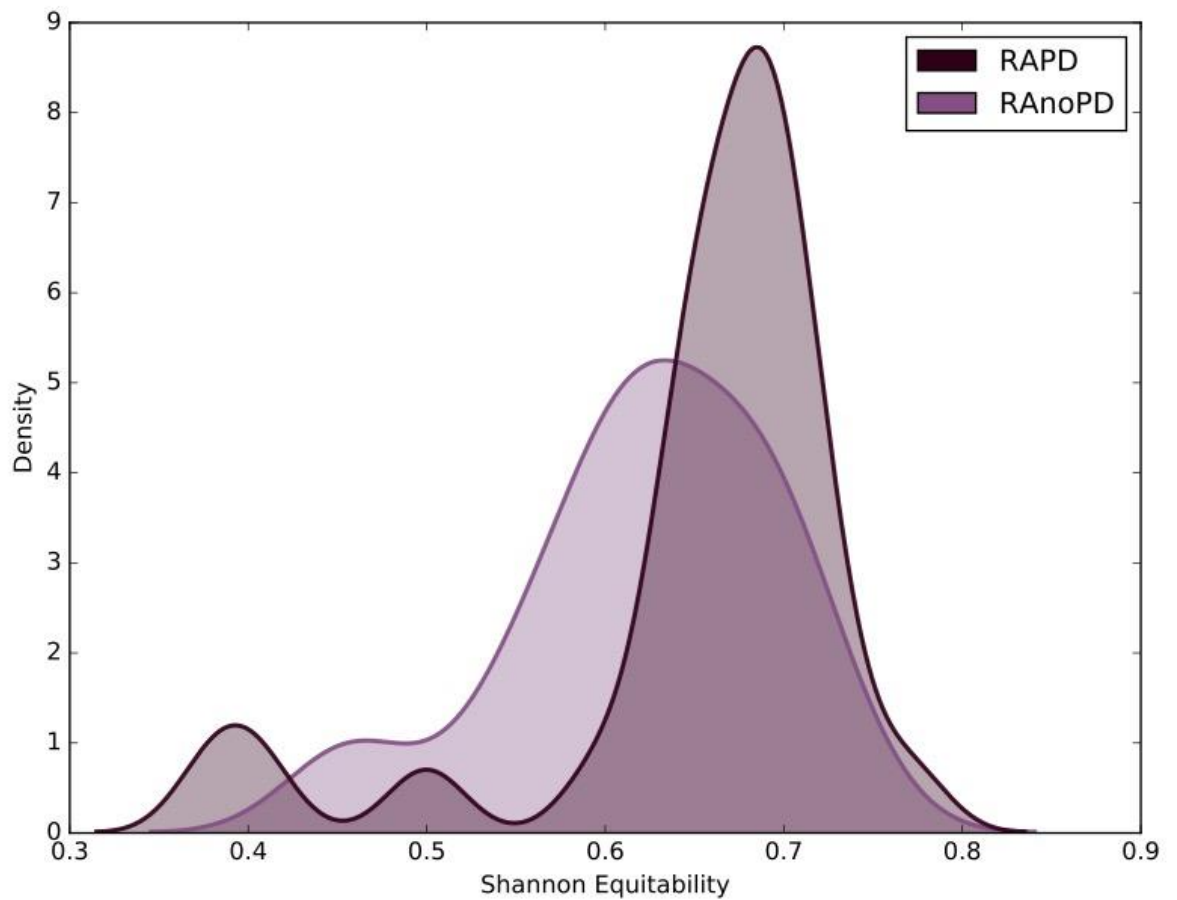


Figure 5. 20 Differences in alpha diversity between rheumatoid arthritis (RA) patients with and without periodontitis (PD). Kernel plots of alpha diversity (Shannon diversity analysis). Test: Mann-Whitney U, significance if  $p < 0.05$ . The peak indicates the median values for each group, and the x-axis shows the data range. n RA no PD=22; RA PD= 26. Methods described in section 2.5

#### 5.6.5. Gram and oxygen characteristics

To investigate what characteristics contribute to differentiate the oral microbiome in RA patients with and without periodontitis, group analysis was performed to investigate if these differences were reflected in the Gram staining characteristics and oxygen requirements of the two communities (Figure 5. 21). While the RAPD group had a higher percentage of Anaerobes compared to the periodontally healthy, only the Gram-positive species were significantly elevated (Mann-Whitney U test,  $p=0.01$ ) and the Gram-positive facultative species were in significantly higher percentage in the periodontally healthy group ( $p=0.008$ ).

While the RA noPD group had a higher percentage of anaerobes compared to the systemically and periodontally healthy group (Figure 5. 9), these results show that the periodontitis condition in RA brings a higher percentage of anaerobes. However, this pattern is not the same as in systemically healthy, as there is a lower percentage of Gram negative anaerobes in RAPD compared to noRAPD and no difference between RAPD and RAnoPD. This corroborates that in RAPD, the predominant bacteria driving the periodontal tissues to disease are different compared to systemically healthy.

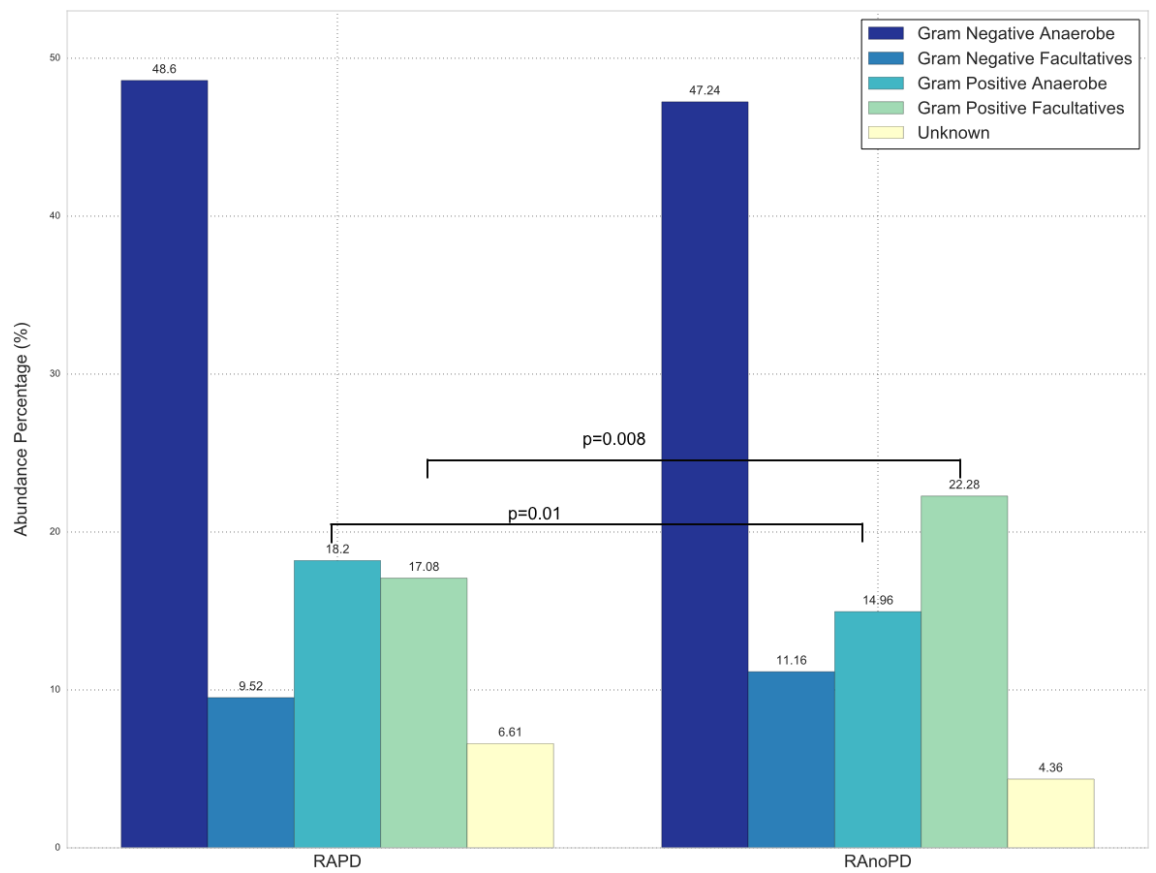


Figure 5. 21 Differences between RAPD and RA noPD patients with periodontitis in abundance of species by gram staining and oxygen requirement characteristics. Mann-Whitney U test, significance if  $p < 0.05$ . Abbreviations: RA, rheumatoid arthritis; PD, periodontitis. Methods described in section 2.5.

#### 5.6.6. Core microbiome

As in the previous sections, the core microbiome (shared by >80% of the patients in the group) was identified. In the RAPD group, 335 OTUs were identified as core bacteria with 34 of those unique for RAPD and not found in the core microbiome of RAnoPD, and with numerous species from the genera *Prevotella*, *Treponema* and *Porphyromonas* were part of this community. In the RAnoPD group, 325 OTUs were identified as core microbiome with 24 of those unique for the core of RAnoPD, numerous species from the *Actinomycetes* (including *A. actinomycetenscomitans*), *Streptococcus*, *Enterococcus* and *Neisseria* genera.

#### 5.6.7. Differences in relative abundance and unique species

A total of 176 OTUs were found to be significantly different in abundance (DESeq2) between the RAPD group and RA no PD (p adjusted value  $p < 0.05$ ). As this could be attributed to the rare species, the different analysis performed between the core species for each group, showed 134 species that were significantly different between the core microbiomes of the two groups. *Porphyromonas gingivalis* was part of the core microbiome of both RAnoPD and RAPD groups with no statistically significant differences in the relative abundance between the groups. *Cryptobacterium curtum*, while it was part of both core microbiomes and was significantly ( $p < 0.048$ ) elevated in the RAnoPD group (mean RAnoPD 28.2, mean RAPD 6.5).

#### 5.6.8. Differences in network correlations

The network correlations from the two groups have been described in section 5.4.7 and 5.3.7. Different network structures are found between the two groups, as a large tight cluster of numerous species inter-correlated form the main node in the RAPD group, no the RA periodontally healthy group a smaller cluster was found. However, the species in both clusters were predominantly Gram negative anaerobes, from the genra Prevotella, Treponema, Porphyromonas, etc. While periodontal health shows a a microbial community a network with lower degree and modularity class, the bacteria that seemed to lead the community assembly in people with RA were similar.

### 5.7. Effect of periodontal therapy in RA microbiome

The change in the subgingival microbiome of RA patients diagnosed with periodontitis before and after periodontal treatment was investigated. Samples were collected at baseline and 3 and 6 months after the hygiene appointment as part of OPERA study (chapter 2). After the baseline appointment, patients were randomised into the treatment group (immediate), which received periodontal therapy, or control group, which received hygiene instructions. 21 patients were randomised in the immediate group and 25 patients in the delayed group. However, due to drop outs or patients that did not give plaque samples in one of the visits, only 41 patients donated plaque samples in the 3 visits (baseline, 3 months after and 6 months after), so some of the analysis included a reduced number of patients.

### 5.8. Clinical characteristics

The effect of periodontal therapy on the clinical characteristics of the immediate group are represented in Table 5. 6. A significant reduction in cumulative probing depth (cumPPD) and BoP was found after 3 and 6 months of periodontal therapy. However, although a significant reduction of pocket depth was achieved 3 months after the periodontal treatment, the significance was lost after 6 months. Although there was a reduction of all the RA parameters after periodontal therapy, these changes were not significant, except for number of tender joints after 3 months.



The baseline comparison between the 2 groups resulted in significant differences in DAS28 and VAS between the 2 groups, with the control group having a higher DAS28 (Mann-Whitney U test,  $p=0.02$ ) and VAS (Mann-Whitney U test,  $p=0.03$ ). The rest of the parameters showed no difference at baseline between the two groups. These differences at baseline were also found after 6 months of therapy (or hygiene instructions for the control group) as significant differences were found in DAS28 (Mann-Whitney U test,  $p=0.009$ ) and VAS (Mann-Whitney U test,  $p=0.04$ ).

Table 5. 6 Clinical parameters in the immediate group before and after periodontal therapy. Test: Wilcoxon paired test, significance if  $p < 0.05$ .

	<b>Immediate group baseline (N=20)</b>	<b>Immediate group visit (N=16)</b>	<b>Immediate group visit 2 (N=15)</b>	<b>Significant Baseline vs V2</b>	<b>Significant Baseline vs V3</b>
<b>CumPPD median (IQR)</b>	67.5 (64)	41.5(47)	44 (47)	0.006	0.03
<b>PPD median (IQR)</b>	3.4 (1.3)	2.9 (0.8)	3.1 (0.9)	0.005	ns
<b>CAL median (IQR)</b>	3.7 (2.02)	3.6 (1.9)	3.8 (2.7)	ns	ns
<b>BoP median (IQR)</b>	0.25 (0.4)	0.15 (0.2)	0.1 (0.3)	0.002	0.01
<b>DAS28 median (IQR)</b>	4.4 (1.6)	3.7 (1.5)	4.3 (2.1)	ns	ns
<b>ESR median (IQR)</b>	14.5 (28)	13.5 (15)	13 (25)	ns	ns
<b>VAS median (IQR)</b>	60 (38)	40 (36)	50 (45)	ns	ns
<b>Tender median (IQR)</b>	7.5 (8)	5.5 (9)	9 (7)	0.03	ns
<b>Swollen median (IQR)</b>	0.5 (2)	0.5 (2)	0 (2)	ns	ns

Table 5. 7 Clinical parameters in the delayed group before and after oral hygiene instructions. Test: Wilcoxon paired test, significance if  $p < 0.05$ .

	<b>Delayed group baseline (N=25)</b>	<b>Delayed group visit 2 (N=25)</b>	<b>Delayed group visit 3 (N=22)</b>	<b>Significant Baseline vs V2</b>	<b>Significant Baseline vs V3</b>
<b>CumPPD median (IQR)</b>	65 (38)	85 (39)	72 (44)	ns	ns
<b>PPD median (IQR)</b>	3.15 (0.6)	3.4 (0.9)	3.3 (1.1)	ns	ns
<b>CAL median (IQR)</b>	3.4 (0.8)	3.6 (1.7)	3.7 (1.3)	ns	ns
<b>BoP median (IQR)</b>	0.24 (0.21)	0.2 (0.4)	0.13 (0.37)	ns	ns
<b>DAS28 median (IQR)</b>	5.4 (1.8)	4.9 (2.6)	4.9 (1.8)	ns	ns
<b>ESR median (IQR)</b>	22 (25)	16 (28)	23 (19)	0.007	ns
<b>VAS median (IQR)</b>	70 (28)	65 (23)	65 (35)	ns	ns
<b>Tender median (IQR)</b>	14 (14)	13 (15)	10 (15)	ns	ns
<b>Swollen median (IQR)</b>	1 (4)	1 (3)	1 (4)	ns	ns

### 5.9. Longitudinal analysis of immediate group: change with periodontal treatment

18 patients from the immediate group donated plaque samples at baseline (V1) and 3 months after treatment (V2). Between V1 and V2, there were significant differences in the PCoA analysis of weighted unifracs distances ( $p=0.03$ ) but not unweighted ( $p=0.8$ ) or Bray Curtis ( $p=0.053$ ), which implied that periodontal treatment changed the microbiome in community structure but not community membership (Figure 5. 22). These changes resulted in a significant reduction in the percentage of anaerobes that were both Gram positive ( $p=0.0003$ ) and Gram negative ( $0.01$ ) and a significant increase of Gram positive facultatives ( $p=0.005$ ).

To further analyze this difference in community structure (group-wise), paired differences were analyzed and 42 OTUs were discovered to significantly change after periodontal treatment (out of 548) with the majority of those changes (28 OTUs) representing a reduction in relative abundance and a minority (14 OTUs) increasing their abundance. Among numerous bacteria from the *Fretibacterium* genera, *Tanerella forsythia* and *Treponella denticola* were significantly reduced after periodontal therapy. *Aggregatibacter actinomycetemcomitans* was reduced non-significantly ( $p=0.06$ ) as well as *Porphyromonas gingivalis* ( $p=0.1$ ).

In total, 19 patients donated plaque samples at both V1 and V3 (6 months after periodontal therapy) and only 11 OTUs were different (pair wise). No significant differences were found either weighted, unweighted or bray curtis distances

matrices, showing that the changes found 3 months after periodontal therapy may be reversed if periodontal therapy is not continued.

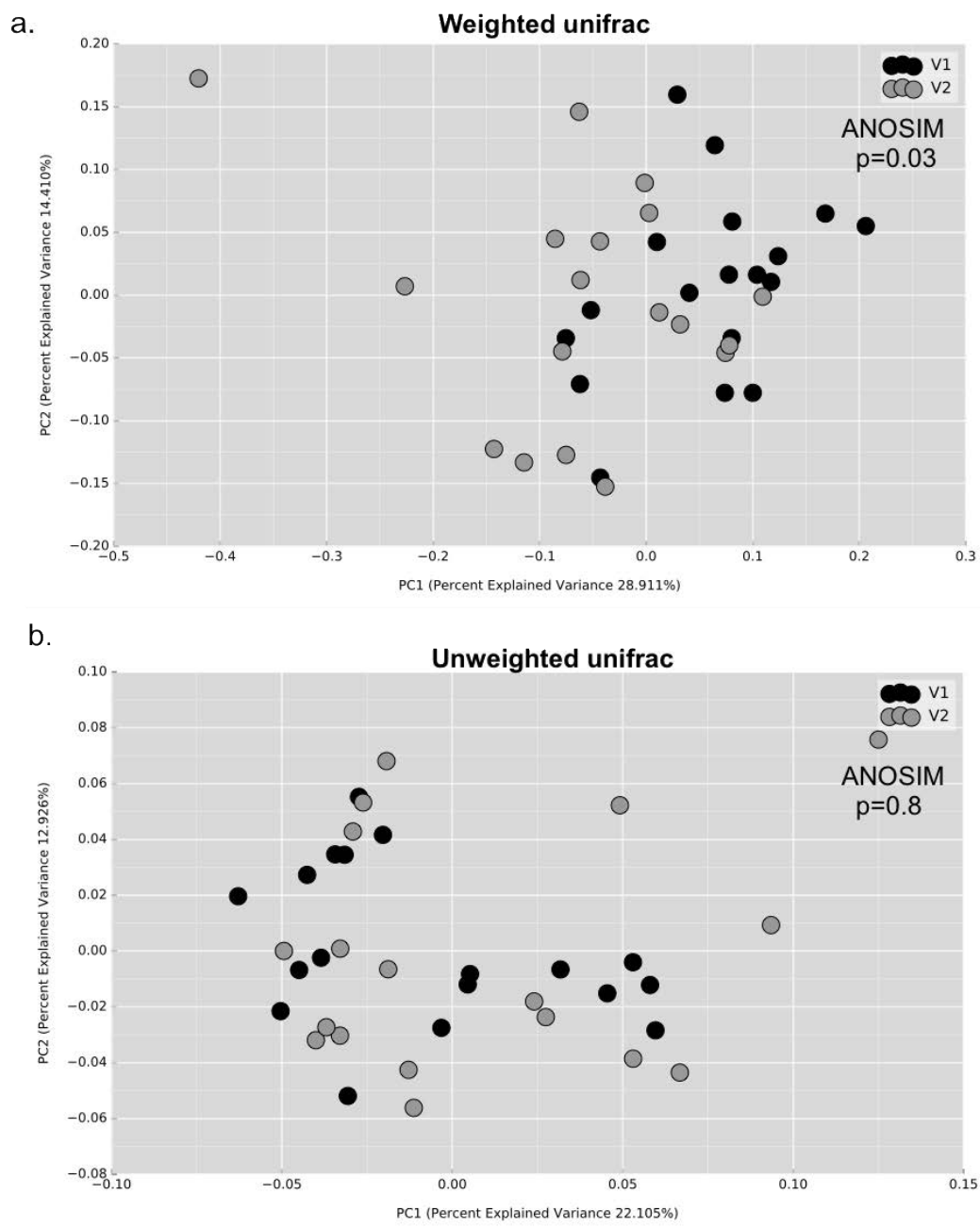


Figure 5. 22 Differences in beta diversity between baseline (V1) and 3 months after periodontal therapy (V2) in RA patients (N=18). Principal Coordinates Analysis (PCoA) plots of unweighted (a.) and weighted (b.) of unifracs distances. Clustering significance tested with ANOSIM test (significance if  $p < 0.05$ ). Methods described in section 2.5.

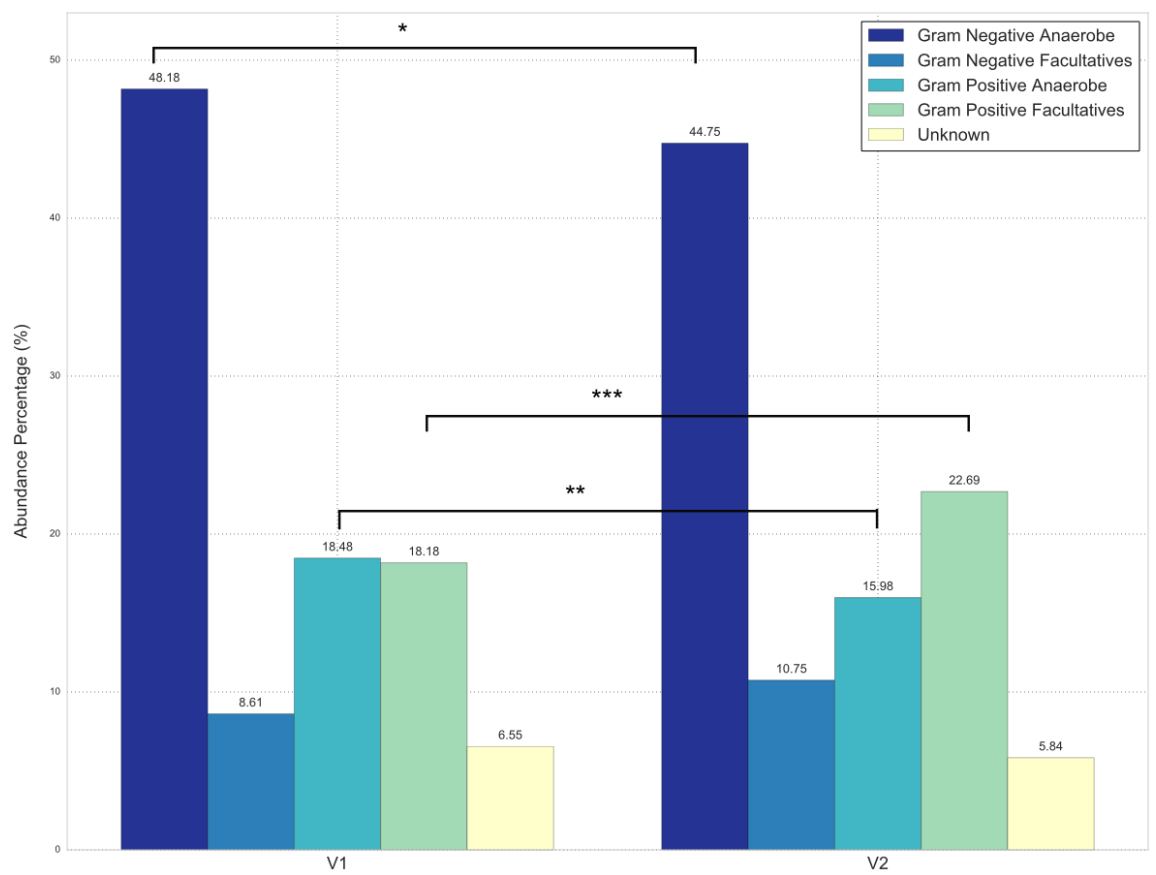


Figure 5. 23 Differences between baseline (V1) and 3 months after periodontal therapy (V2) in RA patients (N=18) in abundance of species by gram staining and oxygen requirement characteristics. Wilcoxon matched-paired signed ranked test, significance(\*) if  $p < 0.05$ (\*\* if  $p < 0.01$ ; \*\*\* if  $p < 0.001$ ).. Methods described in section 2.5.

#### 5.10. Change of relative abundance

Group wise, 42 OTUs changed significantly after periodontal therapy. 40 of these OTUs were part of the core microbiome (present in 80% of patients), which showed that these changes did not only occur in the rare microbiome. 40% of these core OTUs were reduced after periodontal therapy, predominated by species from the *Fretibacterium* genera, *Veillonacea*, *Treponema* and *Tanerella*. The group of bacteria that significantly increases in relative abundance after periodontal treatment is predominated by *Streptococcus*, *Actinomyces* and *Neisseria*.

10 OTUs were found only at baseline and not V2 and 8 OTUs were found only at visit 2, but these OTUs were not part of either core microbiome so those OTUs represented rare species.

#### 5.11. Delayed group

In the control group (25 patients), beta diversity analysis showed no significant differences between microbiome at baseline (V1) and 3 months (V2) (Figure 5. 24) or 6 months after the appointment with the hygienist (V3) and only 3 OTUs changed significantly in a paired-wise analysis of differential abundance (one-sample t-test Bonferroni corrected) after 3 months and 16 OTUs changed significantly after 6 months (out of 560 OTUs in total).



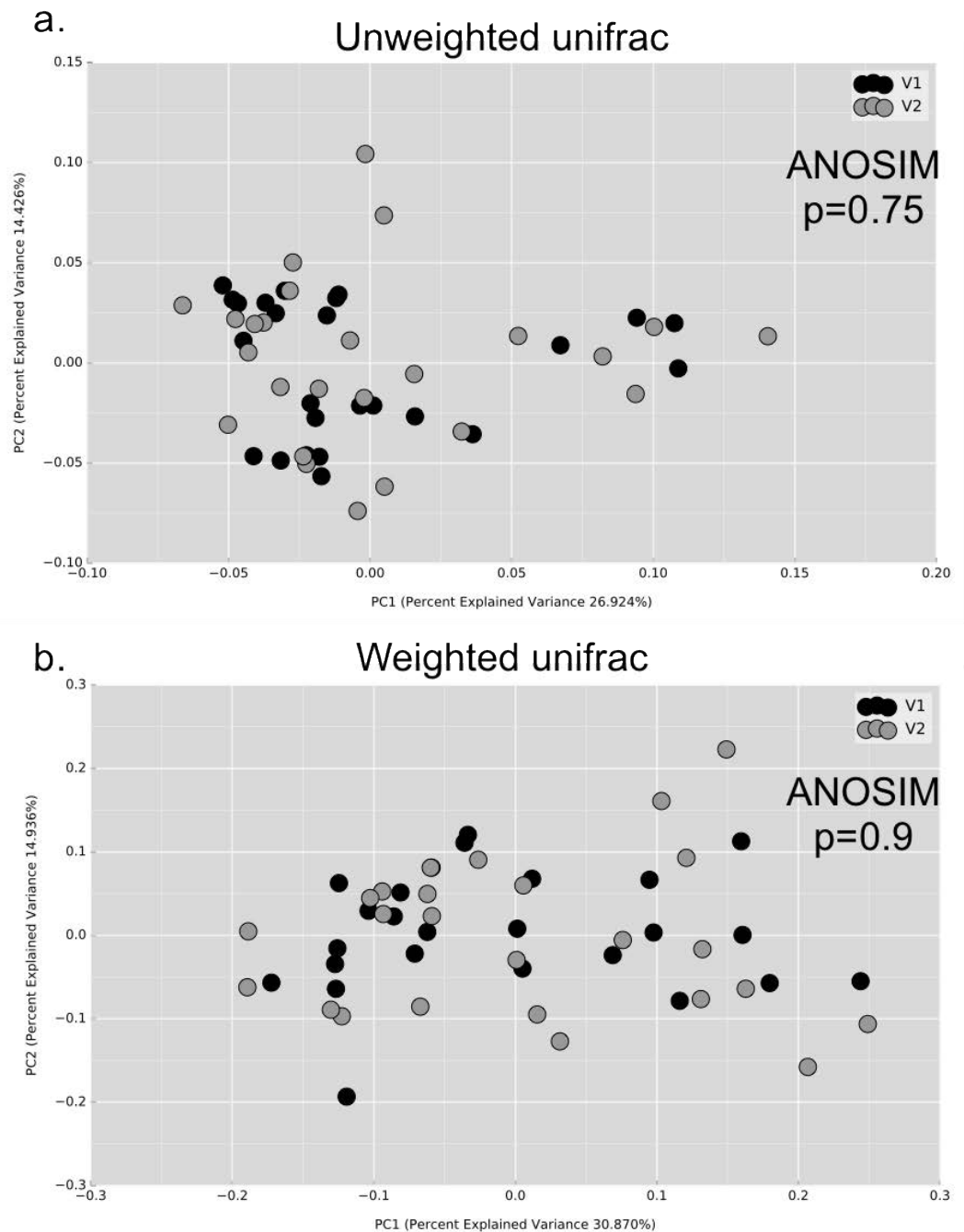


Figure 5. 24 Differences in beta diversity of the control group between baseline (V1) and 3 months after hygiene instructions given (V2) in RAPD patients (N=25). Principal Coordinates Analysis (PCoA) plots of unweighted (a.) and weighted (b.) of unifrac distances. Clustering significance tested with ANOSIM test (significance if  $p < 0.05$ ). Abbreviations: RA, rheumatoid arthritis; PD, periodontitis. Methods described in section 2.5.

## 5.12. Other analyses

### 5.12.1 Effect of periodontitis in systemically healthy microbiome

Clinical characteristics of systemically healthy study volunteers are represented in table Table 5. 2. While in the periodontally healthy groups, samples were collected from the deepest sites, plaque samples from both deep and shallow pockets were collected from the periodontitis group in separate vials, allowing the comparison between healthy and disease sites.

When comparing shallow sites of periodontitis patients and deepest sites of healthy controls significant differences were found in beta diversity analysis by both weighted and unweighted unifrac (Figure 5. 25). However, no differences were found in the distribution of Gram staining or oxygen requirements of the species (Figure 5. 28.a).

When comparing the deep sites of periodontitis patients and the deep sites of healthy controls, differences were found in beta diversity (Figure 5. 26) gram staining and oxygen characteristics (Figure 5. 28.b) and alpha diversity (Figure 5. 27).

The core microbiome of shallow sites comparison showed no unique core OTUs for either groups but 70 OTUs were significantly different in abundance out of 317

shared OTUs in both core microbiomes. This shows that although obvious differences exist between deep sites of periodontitis patients and healthy controls, there are also differences in periodontitis patients even when comparing healthy sites.

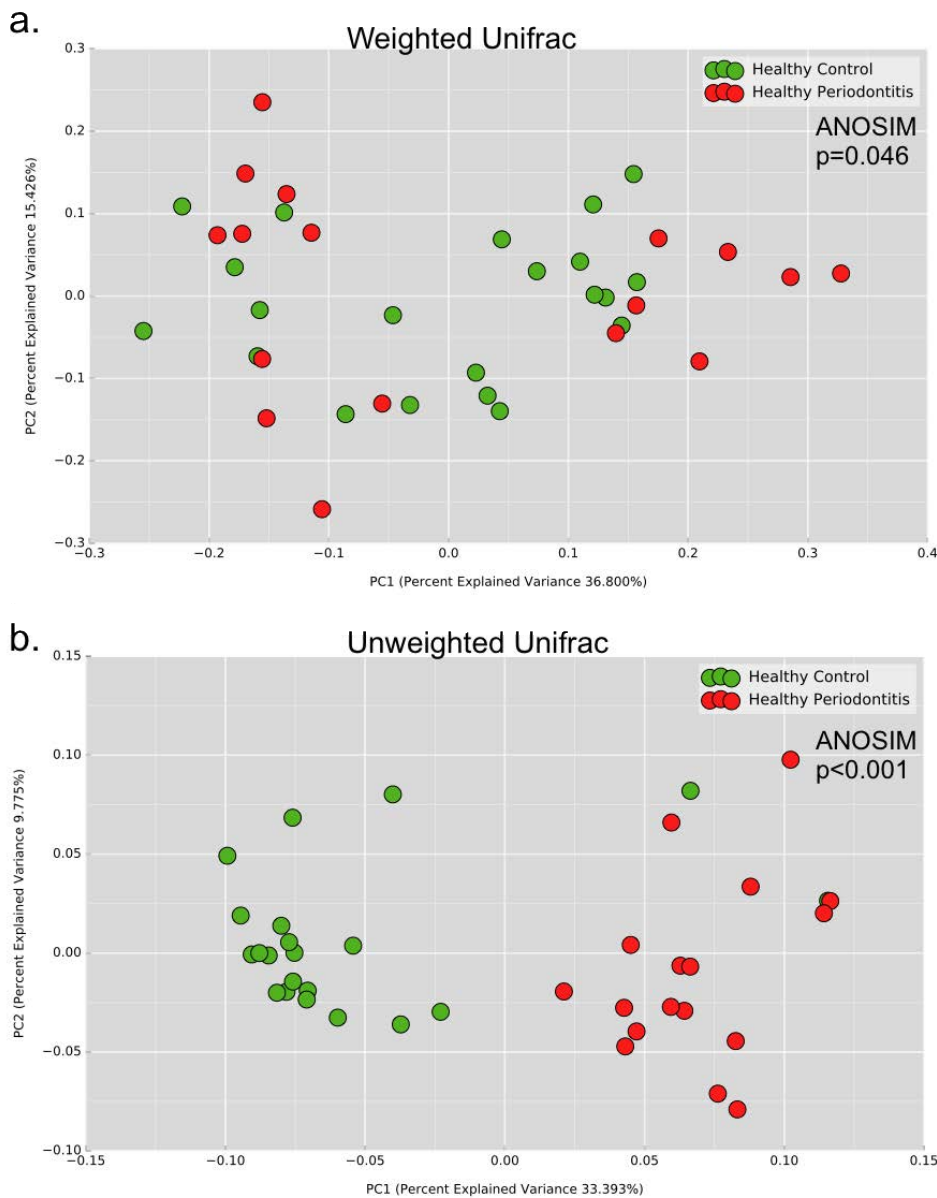


Figure 5. 25 Differences in beta diversity between shallow sites of systemically healthy patients with and without periodontitis. Principal Coordinates Analysis (PCoA) plots of unweighted (a.) and weighted (b.) of unifrac distances. Clustering significance tested with ANOSIM test (significance if  $p<0.05$ ). N Healthy controls=21; n Healthy periodontitis=17. Abbreviations: PD, chronic periodontitis. Methods described in section 2.5.

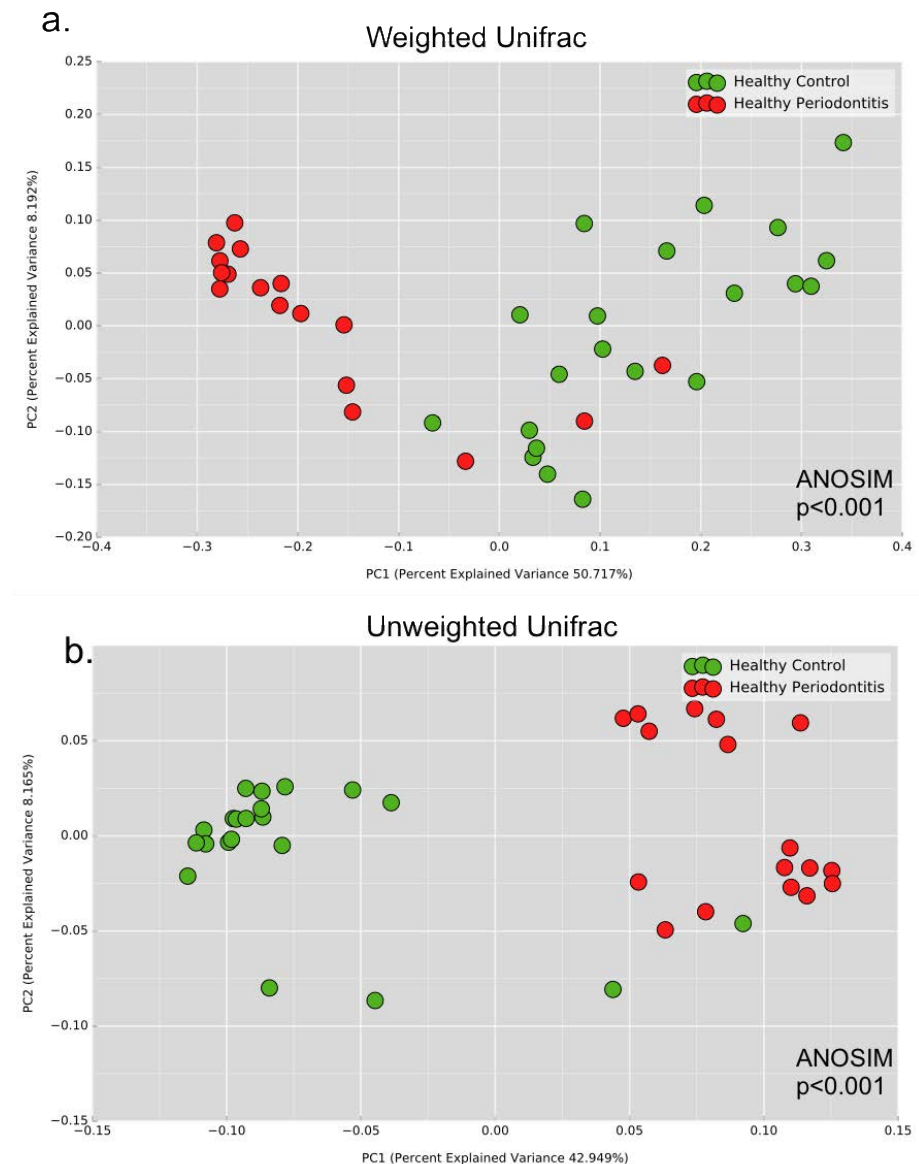


Figure 5. 26 Differences in beta diversity between deep sites of systemically healthy patients with and without periodontitis. Principal Coordinates Analysis (PCoA) plots of unweighted (a.) and weighted (b.) of unifrac distances. Clustering significance tested with ANOSIM test (significance if  $p < 0.05$ ). N Healthy controls=21; n Healthy periodontitis=17. Methods described in section 2.5.

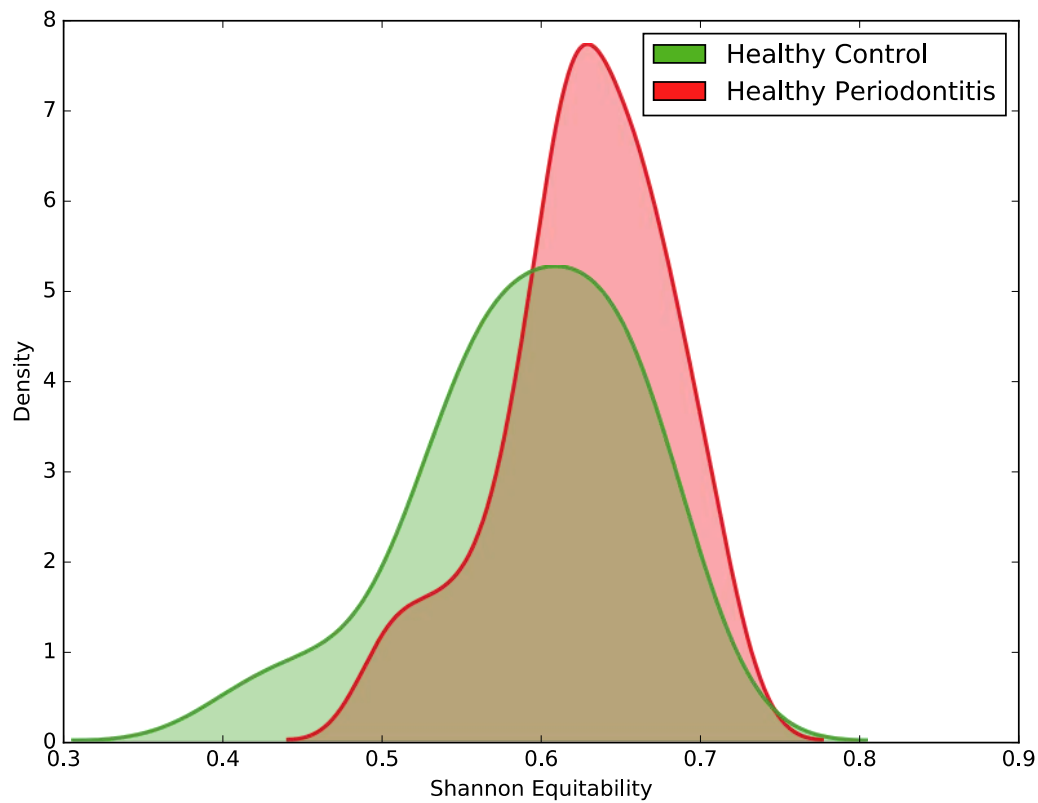


Figure 5. 27 Differences in alpha diversity between deep sites of systemically healthy periodontitis patients compared to controls. Kernel plots of alpha diversity (Shannon diversity analysis). Test: Mann-Whitney U, significance if  $p < 0.05$ . The peak indicates the median values for each group, and the x-axis shows the data range. N Healthy controls=21; n Healthy periodontitis=17. Methods described in section 2.5

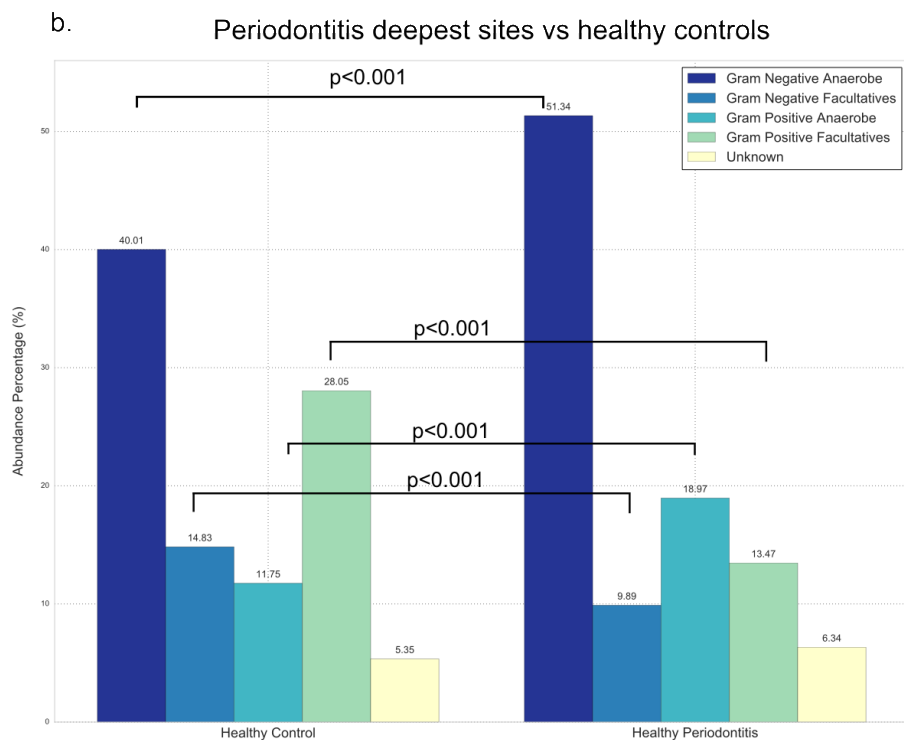
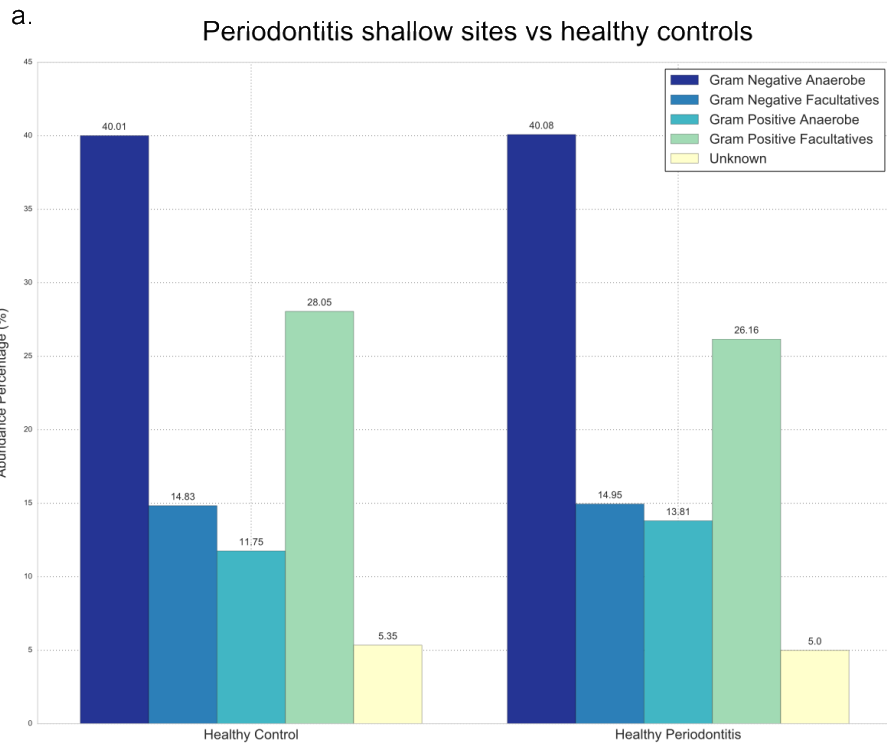


Figure 5. 28 Differences between shallow (a.) or deep (b.) pockets of periodontitis patients and healthy controls in abundance of species by gram staining and oxygen requirement characteristics. Mann Whitney U test, significance if  $p < 0.05$ . Methods described in section 2.5.

### 5.12.2 Microbiome comparison between shallow and deep pockets in noRAPD patients

Further analysis was carried out to investigate the differences between shallow and deep pockets in periodontitis patients (clinical data presented in Table 5. 2). Plaque samples from both shallow and deep pockets were analyzed from 17 periodontitis patients and significant differences were found in the beta diversity analysis of the two groups (Unweighted and weighted unifrac, ANOSIM  $p=0.001$ ).

Paired analysis was conducted and 113 OTUs were found to be different and 24 OTUs were unique for either group (9 unique for deep sites, 16 unique for shallow sites) although none of these unique OTUs were part of the core microbiome, so the unique OTUs represent rare species. Significantly higher percentage of anaerobes (both Gram negative and positive) was found in the deep pockets compared to shallow pockets of the same patients, which was not a surprise as in the deep pockets the conditions are more anaerobic.



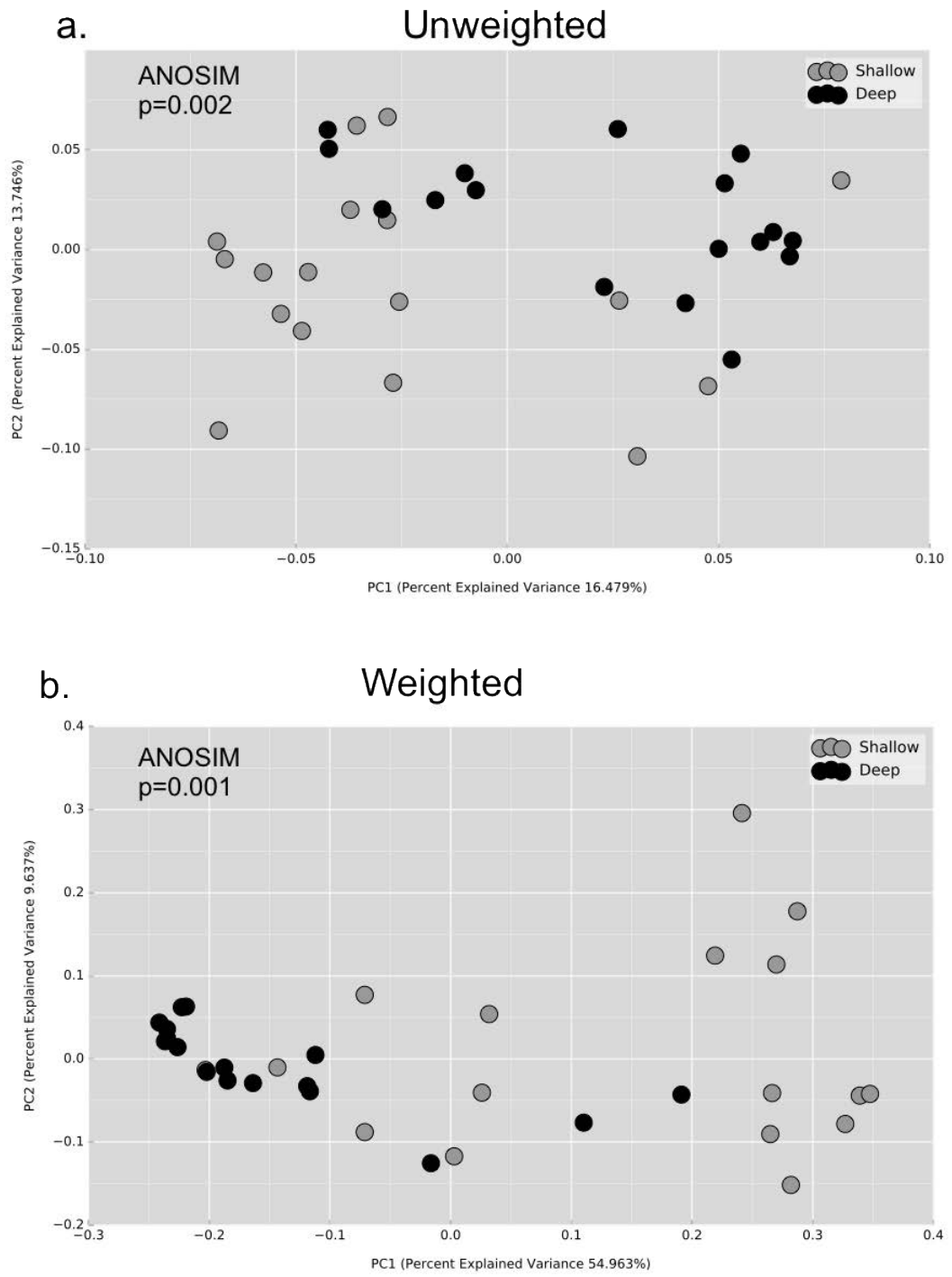


Figure 5. 29 PCoA of unweighted (a) and weighted (b) Unifrac distances in RAPD patients grouped by type of site where plaque was collected (deep vs shallow).  $n = 17$ . Test: ANOSIM, significance if  $p < 0.05$ . Abbreviations: RA, rheumatoid arthritis; PD, chronic periodontitis. Methods described in section 2.5.

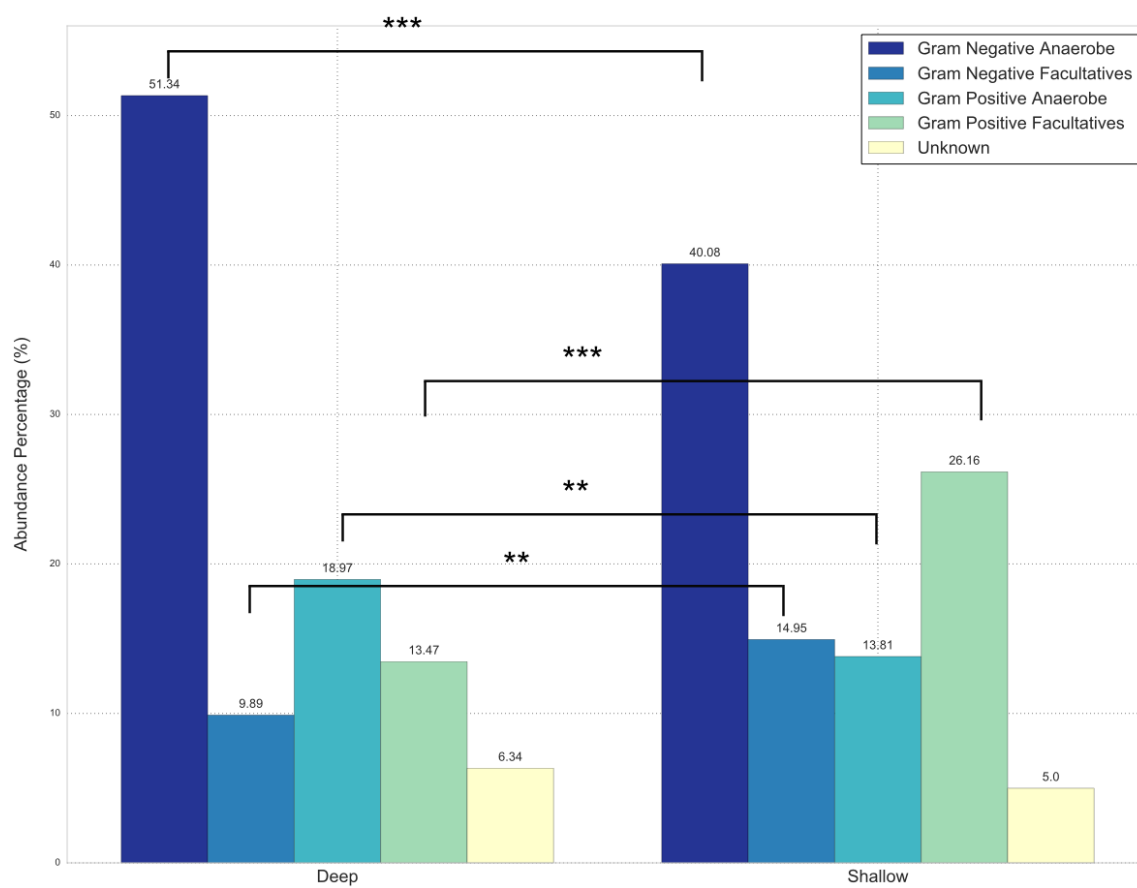


Figure 5. 30 Differences between deep and shallow sites from RAPD patients (N=17) in abundance of species by gram staining and oxygen requirement characteristics. Wilcoxon matched-paired signed ranked test, significance (\*) if  $p < 0.05$  (\*\* if  $p < 0.01$ ; \*\*\* if  $p < 0.001$ ). Methods described in section 2.5.

### 5.13. Curette vs paper point

It has been investigated whether the method of plaque collection determines the type of species identified using molecular microbiological analysis or real-time PCR, showing a strong positive agreement between the two techniques for oral microbiology analysis (Belibasakis, Schmidlin et al. 2014) (Jervøe-Storm, AlAhdab et al. 2007). However, it is not clear in the case of microbiomic analysis. Two plaque samples collected with curette and paper point from the same site in one patient (NoRA noPD) were compared. While beta diversity analysis was not possible, differential expression analysis (DESeq2) was performed and none of the 467 OTUs found in both samples were significantly different between the two collection methods. However, 130 OTUs were unique for one sample compared to the other, these OTUs were rare species not highly abundant (0.47% of relative abundance in the paper point sample and 0.55% of the curette sample). To further investigate the differences, the core microbiome of the two samples (represents 80% of the sample) were compared and no OTUs were unique or significantly different in abundance between the two samples.

From this small analysis we could conclude that both methods could be used in the same analysis, as only a number of OTUs that represent rare species are found through one method of sample collection and not the other.

#### 5.14. Secondary analysis of randomized group: effect of medication

In RA, drug therapy plays an essential role in controlling the inflammation and symptoms. The RA treatment is often a combination of two or more drugs from each type: analgesics, non-steroid anti-inflammatory medication (NSAIDs), glucocorticoids, Disease-modifying anti-rheumatic drugs (DMARDs) and biologic response modifiers (a type of DMARDs). While analgesics and NSAIDs are fairly common and glucocorticoids was one of the exclusion criteria for the OPERA study, the differences between patients with DMARDs therapy and biologics drugs can alter the microbial community composition in the oral cavity (Zhang, Zhang et al. 2015).

For this reason, subgroup analysis was performed from 31 RAPD patients whose medication data was available as part of the longitudinal data from the OPERA study. To compare if the microbiome at baseline was different depending on the type of medication, patients were divided into 3 groups: patients on DMARDs (12 patients), patients on biologics (6 patients) and patients on DMARDs and biologics (13 patients). No significant differences were found (ANOSIM unweighted unifracs  $p=0.5$ ; weighted unifracs  $p=0.4$ ).

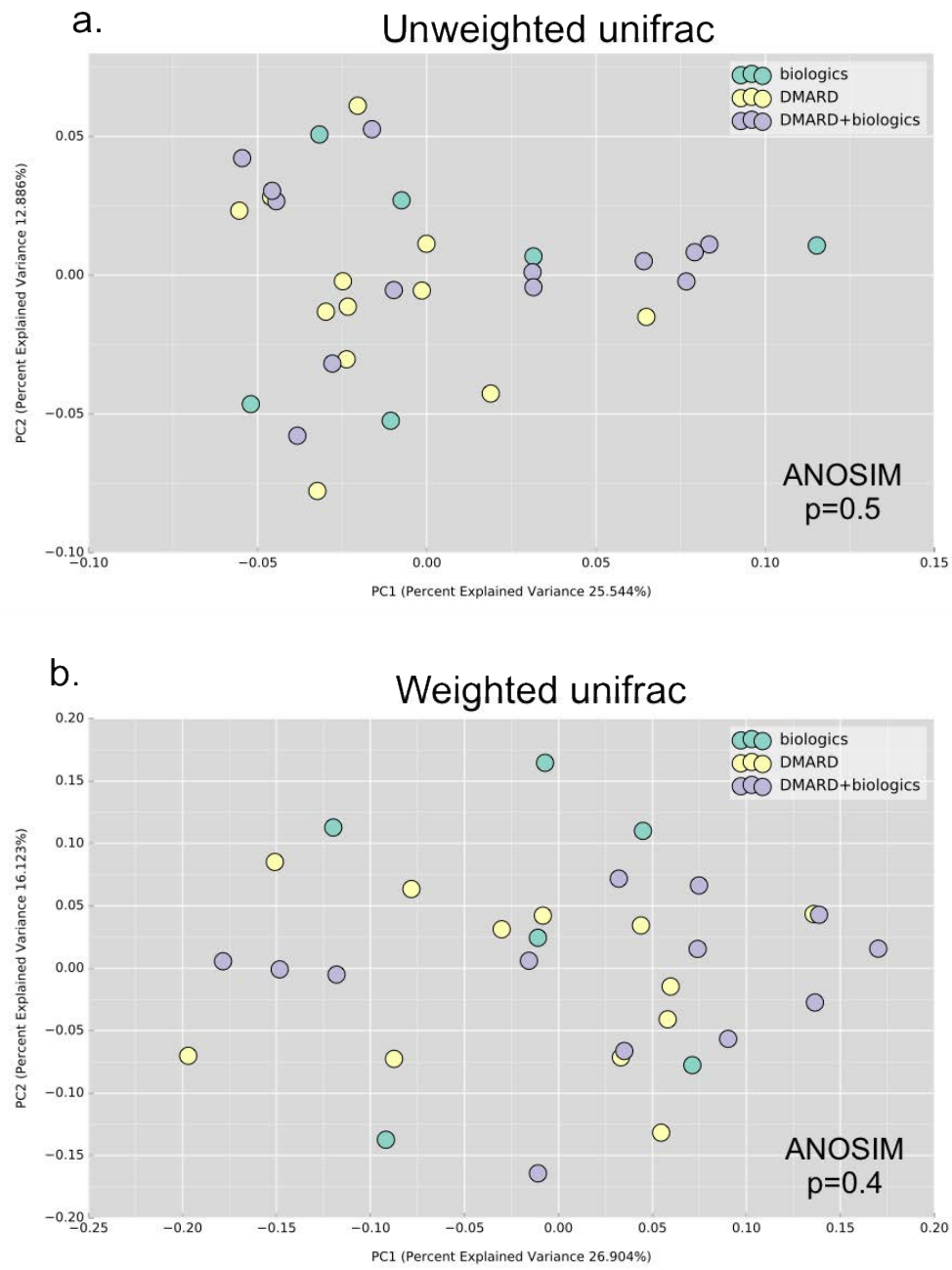


Figure 5. 31 PCoA of unweighted (a) and weighted (b) Unifrac distances in RAPD patients grouped by type of medication. n =31. Test: ANOSIM, significance if  $p < 0.05$ . Abbreviations: RA, rheumatoid arthritis; PD, chronic periodontitis, DMARDs, Disease-modifying anti-rheumatic drugs. Methods described in section 2.5.

## 5.15. Discussion

### 5.15.1. Cross-sectional comparisons of oral microbiome

The results presented in this chapter showed that there are differences in the oral bacteria composition and community structure in RA that is changed with periodontal status. This supports the existence of cross-talk between the immunological condition of RA and the oral microbiome. This cross-talk that results in changes in the conditions of the oral cavity in RA, facilitating the growth of anaerobic bacteria compared to healthy controls. These differences found in the RA microbiome could be due to differences in the clinical characteristics of the population studied, such as the differences in age between the study groups or the RA medication. However, in our study, none of the clinical parameters seemed to explain the shift in the microbiome other than the periodontal and rheumatological features. Nevertheless, other non-RA medication and other comorbidities might have an effect on the oral bacteria and as we did not have such extensive record of patient's comorbidities and medications, it cannot be concluded from our study that these conditions did not alter the oral microbiome and future research is needed to investigate this.

It has been described that the oral microbiome in RA is altered compared to controls (Scher, Ubeda et al. 2012, Zhang, Zhang et al. 2015). However, the lack

of differentiation between periodontally healthy patients and patients with chronic periodontitis in these studies could explain the differences found between the groups, as RA patients have a much higher prevalence of periodontitis compared to the systemically healthy population (Tang, Fu et al. 2017). In section 5.3, the oral microbiome in periodontally healthy RA patients was investigated and significant differences were found compared to controls.

The main hypothesis connecting the oral microbiome and RA is based on the presence of *Porphyromonas gingivalis*. However, studies have found no differences in *P. gingivalis* levels in RA compared to controls (Mikuls, Payne et al. 2014) (Zhang, Zhang et al. 2015) and it is also not elevated in new-onset RA patients (Scher, Ubeda et al. 2012). This was also discovered in this chapter, as it was found that in non-periodontitis patients (noRA<sub>no</sub>PD) *P. gingivalis* was not elevated nor correlated with RA. In non-periodontitis patients, *P. gingivalis* is part of the core microbiome in both RA and NoRA groups but although the abundance is higher in the RA group (0.4 fold<sub>2</sub>), the difference is not significant. Other species such as *P. intermedia* are significantly more abundant in NoRA vs RA (3.5 fold<sub>2</sub>). Another species described to be elevated in RA is *Lactobacillus salivarius* (Zhang, Zhang et al. 2015) but although its abundance is increased in RA, the difference is not statistically significant. The difference between our findings and the literature might be explained by the absence of periodontitis in our study population.

Interestingly, we found that in periodontally healthy patients (RA<sub>no</sub>PD), RA provides a more anaerobic environment in the gingival pocket, facilitating the growth of obligate anaerobes. This could be a pre-clinical phase of periodontitis. However, in periodontitis patients, RA does not contribute towards a more anaerobic environment but on the contrary, the group diagnosed with PD alone (no RA) presented the highest proportion of anaerobes (Figure 5. 32). This is interesting as it shows that periodontal bacteria in RA might be different to the periodontal bacteria in systemically healthy.

Conclusions from the human microbiome project estimate that inter-individual differences are greater than differences between sites of the same area of the body and that the microbiome of individuals is relatively stable in time (Turnbaugh, Ley et al. 2007). This is supported by our research, which founds a stable microbiome showing no major changes after 3 and 6 months in the control group, and no major differences between the shallow and deep pockets. However, this contradicts a recent study that shows big differences between shallow and deep sites of periodontitis patients, although they only investigate 9 subjects (Turnbaugh, Ley et al. 2007). More research is needed to elucidate the differences between deep and shallow sites in periodontitis with bigger sample sizes and robust bioinformatic methodologies.



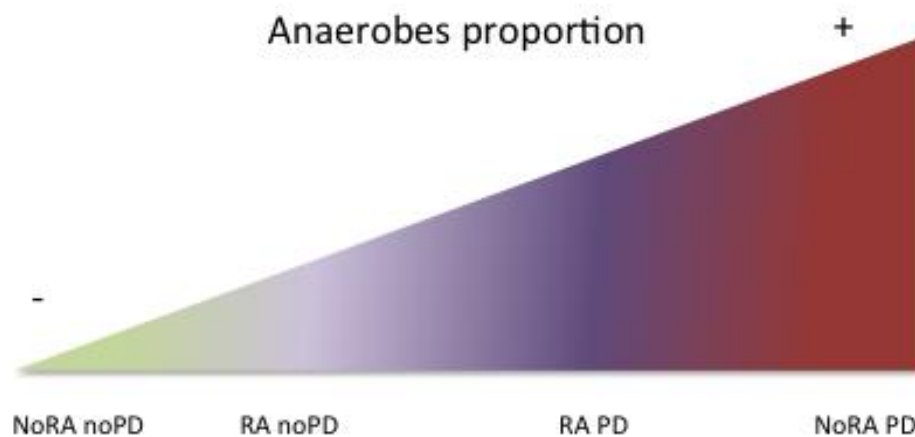


Figure 5. 32 Anaerobes proportion comparison between the 4 study groups.

One of the most intriguing findings was the identification of *Cryptobacterium curtum* as a predominant member of the RA-influenced periodontal microbiome. This Gram-positive, assacharolytic, anaerobic rod (which was previously classified as *Eubacterium saburreum*) degrades arginine through the arginine deiminase pathway and produces substantial amounts of citrulline, ornithine and ammonia (Uematsu, Sato et al. 2006). More importantly, *C. curtum* is enriched in the oral and gut RA microbiomes and in early RA cases (Scher, Ubeda et al. 2012, Zhang, Zhang et al. 2015). Furthermore, early investigations have demonstrated that 48% of the variation in alveolar bone loss in RA patients could be attributed to an organism that was then identified as *E. saburreum* and later reclassified as *C. curtum* (Uematsu, Sato et al. 2003). In the present investigation, this species was a member of the core microbiome in RA patients, demonstrated a 100-fold greater abundance in this cohort when compared to controls with a 39-fold greater odds of detection in this cohort. While this unusually high association

does not necessarily suggest an aetiopathogenetic role for *C.curtum*, this organism is a candidate for further studies.

However, although 16S rRNA is an accepted method to investigate the oral microbiome, there are limitations, as the taxa are predicted based on associations of the 16S gene sequences with taxa defined as OTUs. Therefore, this method is valid to analyze microbiome at phyla or genera level but can lose precision at species level. Whole genome shotgun sequencing has been shown to have numerous advantages over 16S detecting at bacteria species and an enhance capacity to detect diversity (Ranjan, Rani et al. 2016).

In summary, our data suggest that rheumatoid arthritis plays a major role in shaping the oral microbiome, enriching this environment for inflammophilic organisms and those capable of producing substantial amounts of citrulline. Our findings lend further credence to a link between the oral microbiome and RA; however, the directionality or causality cannot be explored with this cross-sectional study design.

#### 5.15.2. Longitudinal comparison: effect of periodontal therapy

In this group of RAPD patients, there was no effect of periodontal therapy in the RA parameters such as DAS or ESR, and the reduction observed in the other chapters (Chapter 3,4 and 6) did not occur. While these differences were surprising it could be explained as, although these patients came from the same trial, not all patients donated the same type of samples for different reasons (some patients had difficult veins, others had to leave before we collected the

plaque, etc) and there were 11 patients which samples were not included for both experiments. In such small sample sizes (<20), 11 patients can drastically change the group outcomes. 15% of the patients that donated bloods for the Chapter 3 and 4 were not in part of the cohort presented in this chapter and 27% of the patients included for the microbiome analysis were not part of the cohort included in chapters 3 and 4. That could explain the difference between the longitudinal outcomes between the chapters.

Even with such small sample size, the periodontal parameters and beta diversity of plaque structure were significantly reduced 3 months after periodontal therapy, leading to a lower proportion of Gram negative anaerobes. These differences were only significant considering the abundance of the species present (community structure) and not the absence-presence (community composition).

However, after 6 months, these periodontal parameters non-significantly increased, and the differences between baseline and 6 months after are not significant for beta diversity or periodontal parameters. This is supported by previous microbiological findings showing that a single course of scaling and root planning is insufficient as periodontal bacteria recolonized the periodontal pocket after 2 months (Sbordone, Ramaglia et al. 1990). The results from this chapter shows that the oral microbiome related to periodontitis and the clinical characteristics return in RA patients so periodontal maintenance treatment should be provided.

In this chapter, we show that periodontal therapy, in RA, is effective in changing the abundance of the species, but not the composition of the biofilm that causes disease. Nevertheless, if periodontal care is not continued with oral hygiene and periodical visits to the dentist, the community structure can return to a disease state and therefore worsen the clinical parameters of disease.

## **6. RESULTS - CHANGES IN GCF CYTOKINES AFTER PERIODONTAL THERAPY**

## 6.1. Introduction

The hypothesis of the study presented in this chapter was that, in patients with rheumatoid arthritis and periodontitis undergoing periodontal therapy there are changes in the GCF cytokine profile, correlated with systemic markers of RA disease and periodontal bacteria.

The aims of this chapter were:

- 1) To compare the level of GCF cytokines before and after periodontal therapy compared to a control group that did not receive such therapy.
- 2) To analyse the correlations between the local cytokine response changes and systemic markers of RA inflammation and periodontal bacteria.

This chapter presents the results from a commercially available multiplex bead-based assay designed to semi-quantify multiple cytokines. Gingival crevicular fluid (GCF) was used to measure 27 cytokines in RA patients with periodontitis collected before and 6 months after randomisation (Table 6. 1). Patients that fell into the immediate group received non-surgical periodontal therapy and the delayed group received oral hygiene instructions. Methods are described in detail in chapter 2.4.

Table 6.1 Cytokines investigated in this chapter.

IL-1 $\beta$	IL-10	IFN- $\gamma$	IL-5	IL-17A	MIP-1 $\beta$
IL-1ra	IL-12 (p70)	IP-10	IL-6	Eotaxin	PDGF- BB
IL-2	IL-13	MCP-1 (MCAF)	IL-7	FGF basic	RANTES
IL-4	IL-15	MIP-1 $\alpha$	IL-8	G-CSF	TNF
			IL-9	GM- CSF	VEGFs

## 6.2. Study volunteers

Patients with periodontitis and active RA were selected for the OPERA longitudinal study (exclusion/inclusion criteria described in Chapter 2.1) and GCF from 44 patients were analysed in this chapter. From the 44 eligible patients, 22 patients were randomly allocated in the immediate group and 22 in the delayed group. Clinical and demographical characteristics of the study population are described in chapter 3, with the exception of two patients included in the immediate group in this chapter, which donated GCF samples but not serum. Differences in clinical parameters at baseline between immediate and delayed group were not significant (Table 3.2 section 3). A significant reduction of periodontal and some of the RA parameters was achieved after periodontal therapy (Table 3.3).

## 6.3. Longitudinal analysis

### 6.3.1. Effect of periodontal therapy on cytokine production

A panel of 27 cytokines was selected, including Th1 and Th2 cytokines (interleukin-2 [IL-2] and gamma interferon [IFN- $\gamma$ ]), pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, and granulocyte-macrophage colony-stimulating factor [GM-CSF]), and chemokines (IL-8).

Calibration curves for each cytokine were interpolated to calculate the concentration of the cytokines studied. Blank values were subtracted from the raw data and results were presented as Arbitrary Units per ml (AU/ml).



The longitudinal analysis of the GCF cytokines in the immediate group revealed a significant decrease of IL8, IL9, VEGF, MP1- $\alpha$  after periodontal therapy that did not occur in the control group. Other cytokines decreased close to significance ( $p < 0.06$ ) after periodontal treatment, such as IL-10, IL-12 and TNF and RANTES. Decreases close to statistical significant could be of interest in studies with such small sample size ( $n=22$ ).

Interestingly, there was a significant increase of cytokines IL-2 and IL-15 in the treatment group after periodontal therapy, which did not occur in the control group.

The rest of the cytokines studied, including IL-1 ( $\alpha$  and  $\beta$ ), IL-6 and IL-17 non-significantly decreased after periodontal therapy and, in the control group, no significant changes were found in any of the 27 cytokines studied, in GCF collected 6 months after randomization.

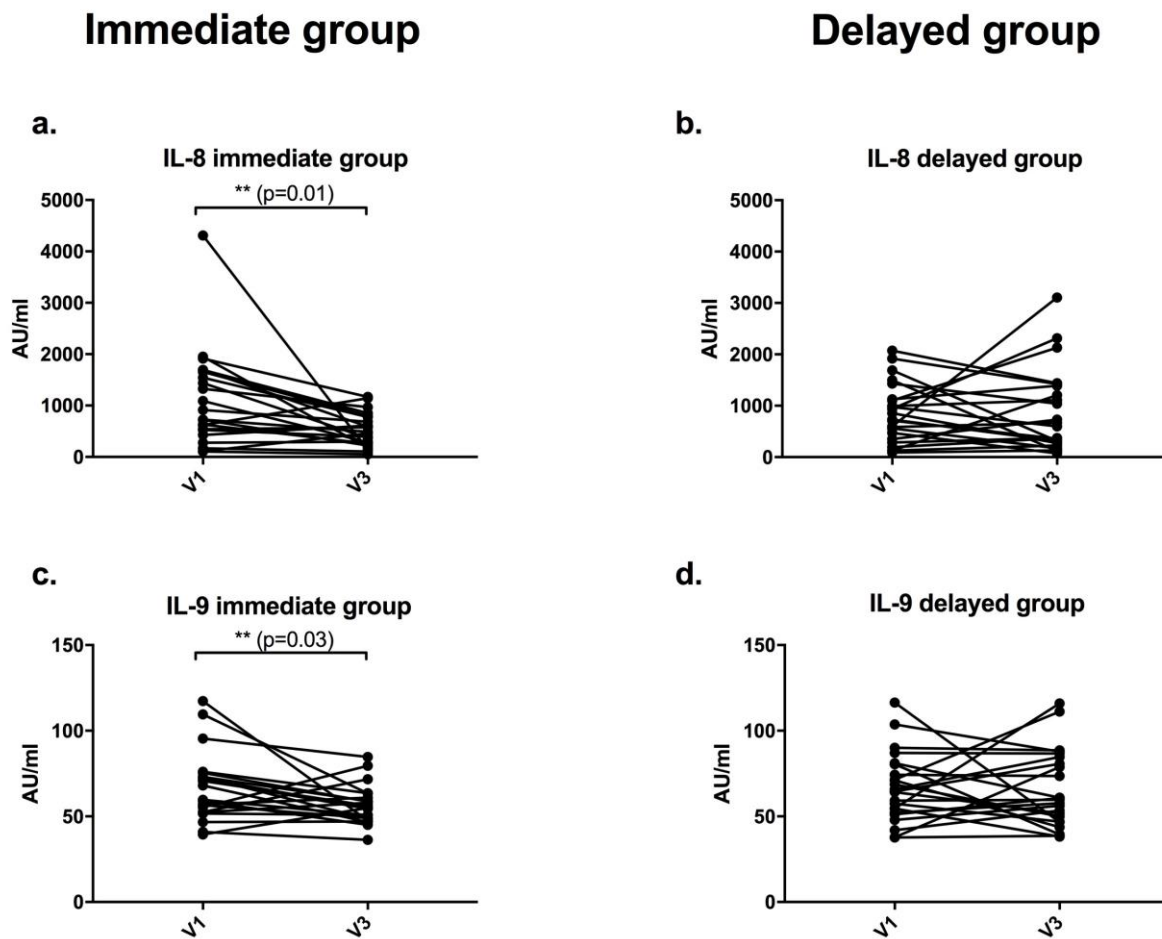


Figure 6. 1 Longitudinal analysis of the cytokines that significantly decrease after periodontal therapy. V1 represents antibody levels in GCF collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml). n Immediate treatment group=22; n Delayed group=22. Statistical test used was Wilcoxon matched pair test (significance \*  $p<0.05$ ; \*\*,  $p<0.01$ ). Methods described in section 2.2. Abbreviations: GCF, gingival crevicular fluid; IL, interleukin.

## Immediate group

## Delayed group

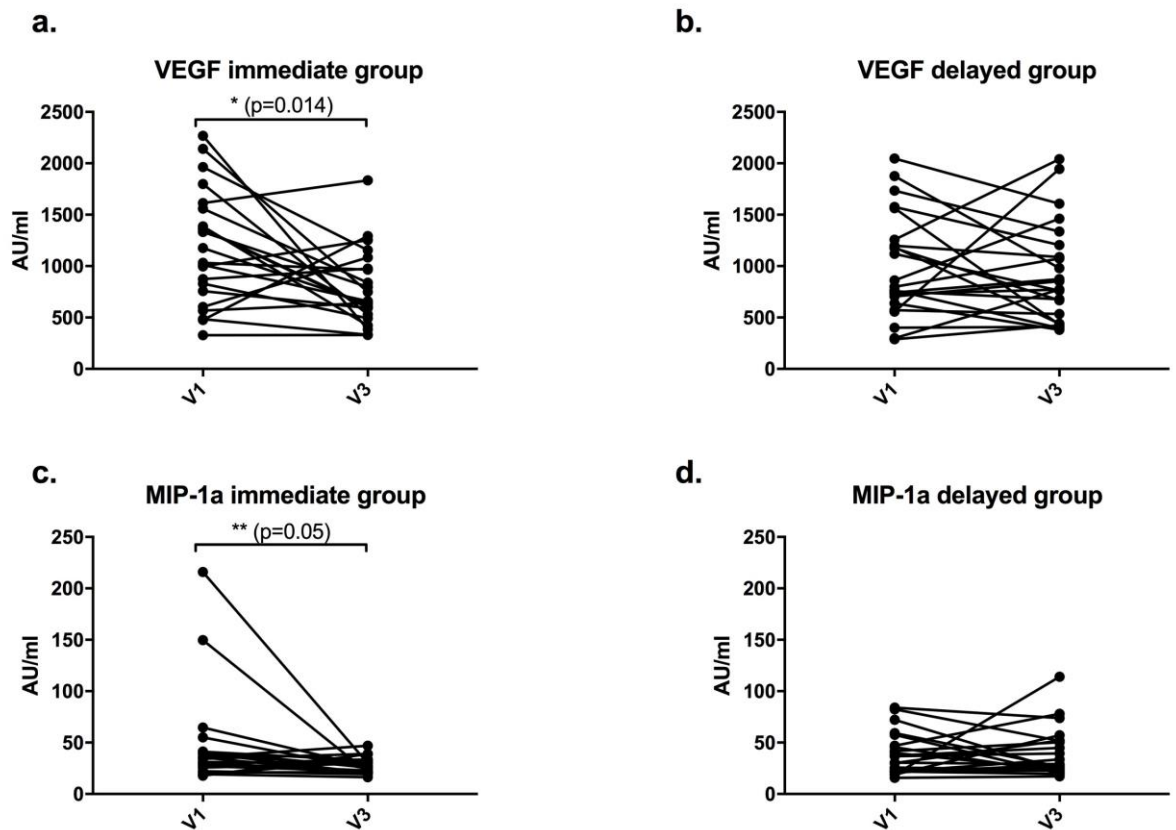


Figure 6. 2 Longitudinal analysis of the cytokines that significantly decrease after periodontal therapy. V1 represents antibody levels in GCF collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml). n Immediate treatment group=22; n Delayed group=22. Statistical test used was Wilcoxon matched pair test (significance \*  $p<0.05$ ; \*\*,  $p<0.01$ ). Methods described in section 2.2. Abbreviations: GCF, gingival crevicular fluid; VEGF, .vascular endothelial growth factor; MIP, macrophage inflammatory protein.

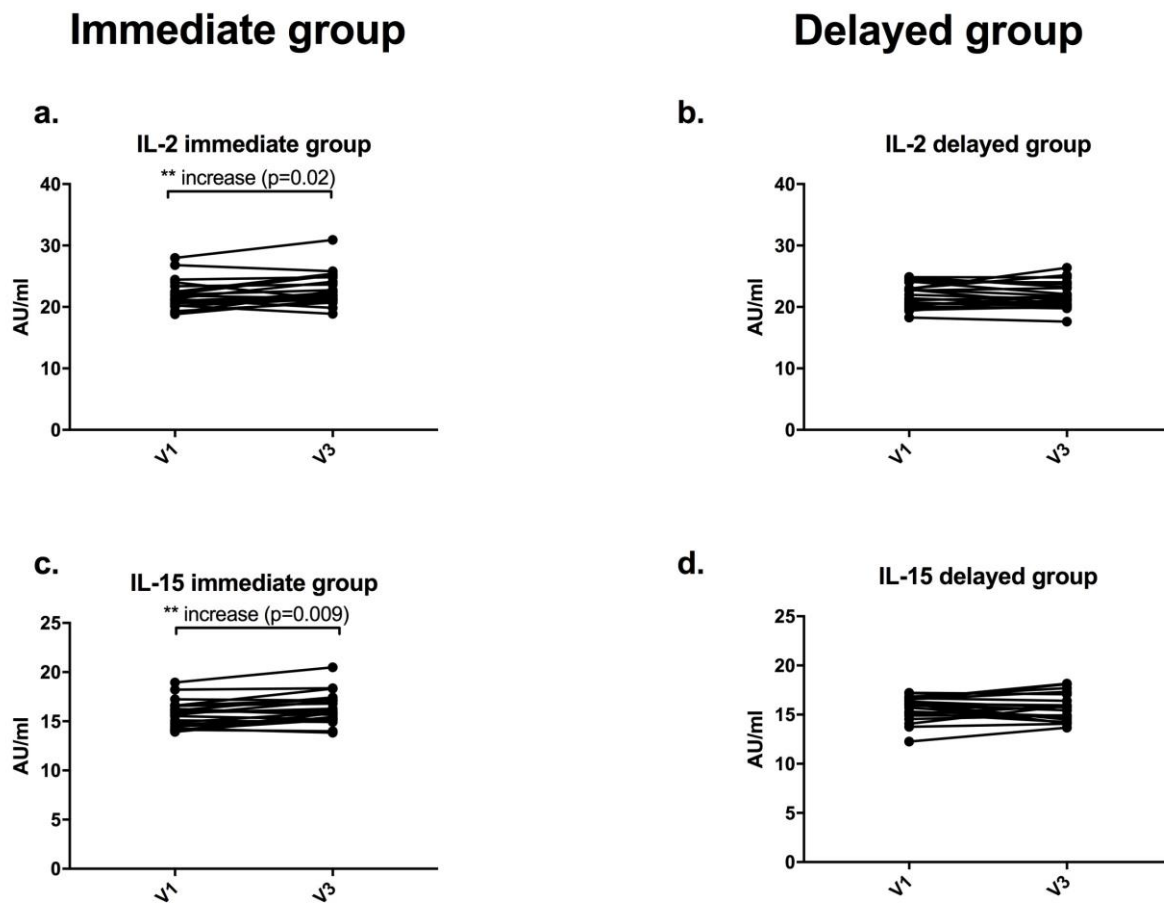


Figure 6. 3 Longitudinal analysis of cytokines that significantly increase after periodontal therapy. V1 represents antibody levels in GCF collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml). n Immediate treatment group=22; n Delayed group=22. Statistical test used was Wilcoxon matched pair test (significance \*  $p<0.05$ ; \*\*,  $p<0.01$ ). Methods described in section 2.2. Abbreviations: GCF, gingival crevicular fluid; IL, interleukin.

## Immediate group

## Delayed group

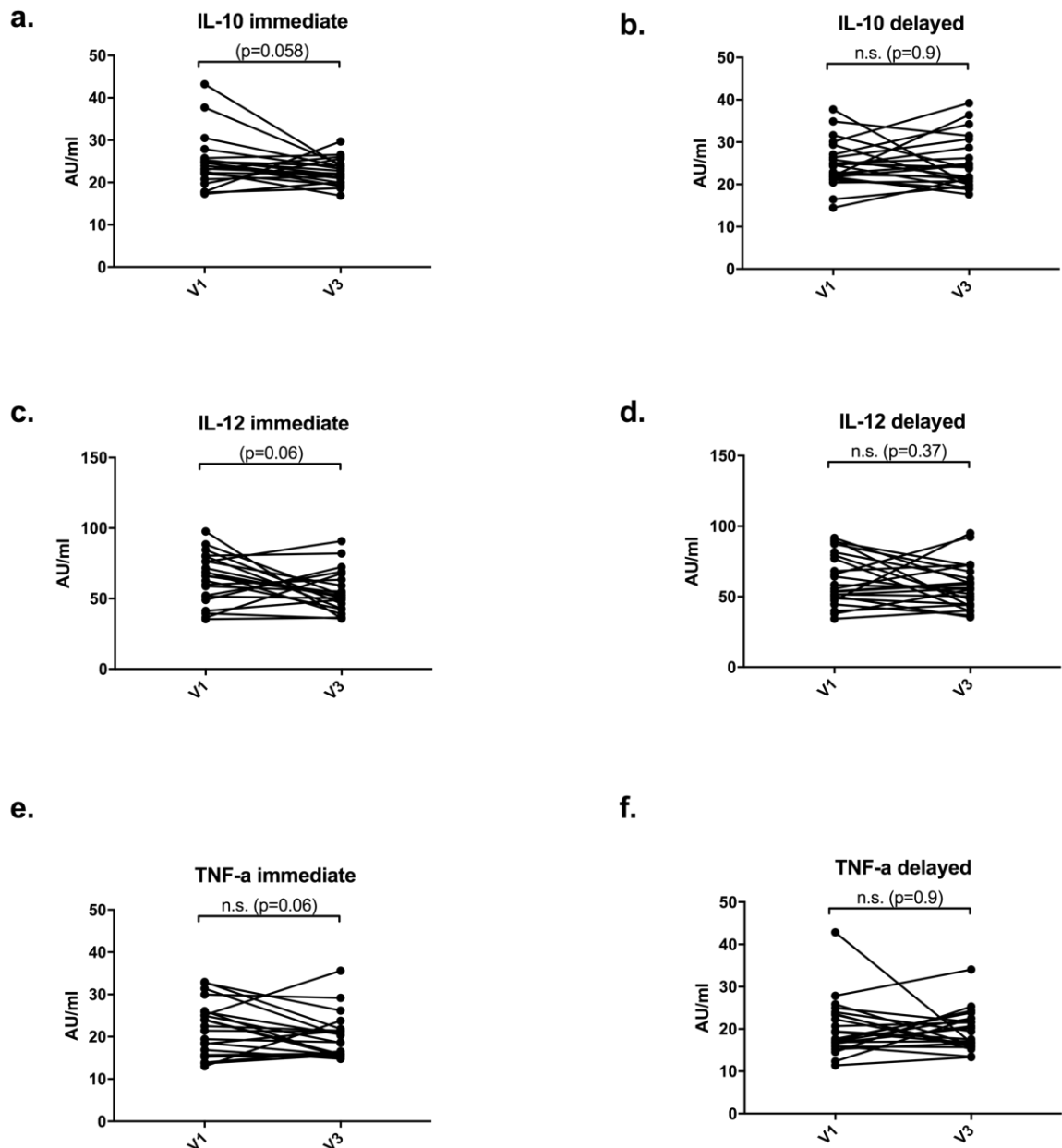


Figure 6. 4 Longitudinal analysis of cytokines that decreased close to significance ( $p \leq 0.06$ ) after periodontal therapy in the immediate group. V1 represents antibody levels in GCF collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml). n Immediate treatment group=22; n Delayed group=22. Statistical test used was Wilcoxon matched pair test (significance \*  $p < 0.05$ ; \*\*,  $p < 0.01$ ). Methods described in section 2.2. Abbreviations: GCF, gingival crevicular fluid; IL, interleukin; TNF, tumor necrosis factor.

#### 6.3.1.1. Effect of periodontal therapy on cytokine production according to smoking and ACPA status

In ACPA positive patients, there was a significant decrease of IL-1b, IL-8, IL-9, IL-10, IL-12, VEGF and TNF and significant decrease of IL-15 after periodontal therapy. The cytokines that revealed significant changes only in ACPA+ patients but not when investigating totality of RA patients were IL-1b, IL-10, IL-12, VEGF and TNF, which, in the main longitudinal analysis, showed p values close to significance. No significant differences were found in the ACPA- patients probably due to the small sample size (N=4).

Table 6. 2. Significant changes in cytokine production after periodontal therapy in ACPA+ patients. Positive values in median of differences represent increase, negative values represent decrease. Test: Wilcoxon paired test, significance  $p < 0.05$

	<b>Median of differences (AU/ml)</b>	<b>P value</b>
<b>IL-1b</b>	-382.055	0.009
<b>IL-8</b>	-388.79	0.014
<b>IL-9</b>	-8.78	0.029
<b>IL-10</b>	-2.35	0.049
<b>IL-12</b>	-12.64	0.006
<b>IL-15</b>	4.34	0.003
<b>VEGF</b>	-330.05	0.001
<b>TNF</b>	-2.31376	0.05

As smoker individuals have been shown to have an increased release of pro-inflammatory cytokines in the GCF (Giannopoulou, Kamma et al. 2003), subgroup analysis stratified by smoking status was performed, which revealed that in smokers, only IL-4 decreased significantly ( $p=0.027$ , median of differences  $-2.27$ ) after periodontal therapy although the small number of current smoker patients ( $N=4$ ) might be the reason for the lack of significances in the rest of the cytokines. Interestingly, it has been shown that although IL-4 is elevated in health, this cytokine is also significantly correlated with smoking (Giannopoulou, Kamma et al. 2003).

In non-smoker patients, there was a significant decrease of IL-1b, IL-12, IL-8 and VEGF, and a significant increase in IL-15 after periodontal therapy. IL-1b and IL-12 appear as significant in this subgroup analysis while it was not significant in the analysis of the whole treatment group (Table 6.3).



Table 6. 3 Significant changes in cytokine production after periodontal therapy in non-smoker patients. N= 13. Positive values in median differences represent increase, negative values represent decrease. Test: Wilcoxon paired test, significance  $p < 0.05$

	<b>Median of differences</b>	<b>P value</b>
<b>IL-1b</b>	-586.06	0.045
<b>IL-8</b>	-247.38	0.037
<b>IL-12</b>	-10.73	0.009
<b>IL-15</b>	0.43	0.013
<b>VEGF</b>	-319.60	0.001

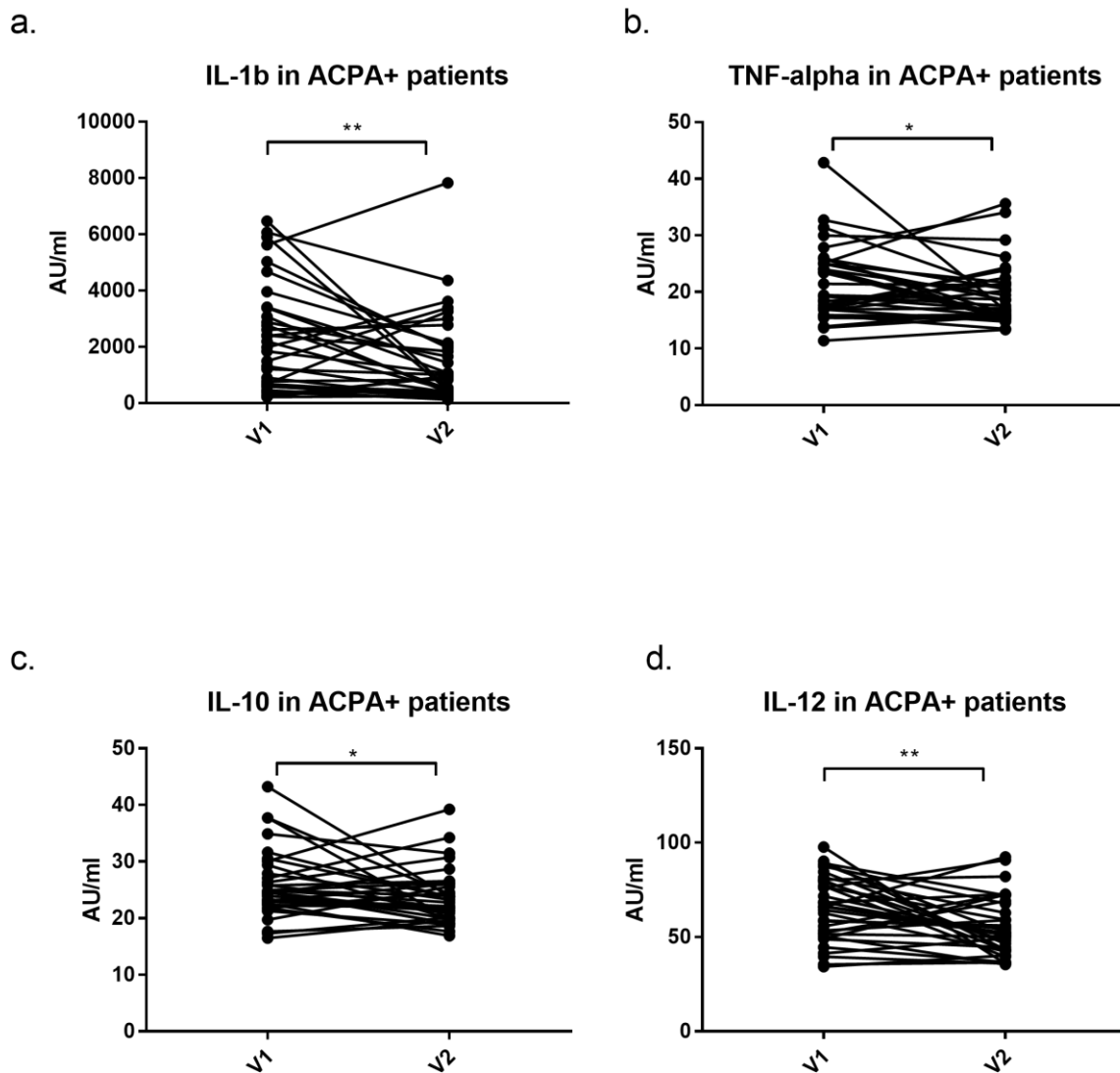


Figure 6. 5 Cytokines that significantly decreased after periodontal therapy in ACPA+ patients only. after periodontal therapy. N= 18. V1 represents antibody levels in GCF collected at baseline and V2 collected 6 months after. Data presented in Arbitrary units per ml (AU/ml). Statistical test used was Wilcoxon matched pair test (significance \*  $p < 0.05$ ; \*\*,  $p < 0.01$ ). Methods described in section 2.2. Abbreviations: GCF, gingival crevicular fluid;

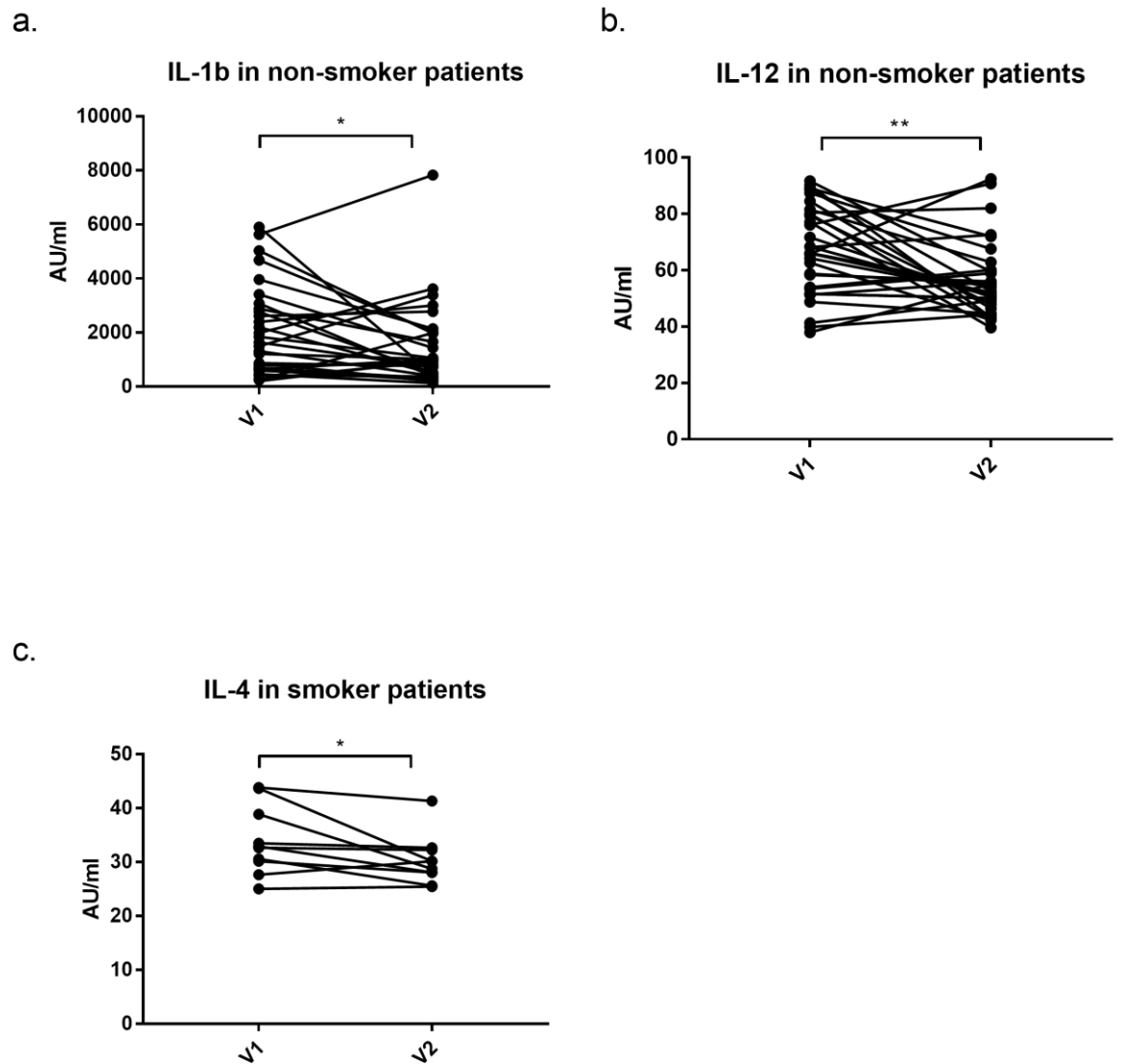


Figure 6. 6 Cytokines that significantly decreased after periodontal therapy when stratifying by smoking status only. V1 represents antibody levels in serum collected at baseline and V2 collected 6 months after. Data presented in arbitrary units per ml (AU/ml). N Treatment group=23; n Delayed group=22. Statistical test used was Wilcoxon matched pair test (significance \*  $p < 0.05$ ; \*\*,  $p < 0.01$ ). Methods described in section 2.2

#### 6.4. Correlation analysis

The Spearman rank correlation analysis was performed to investigate the relationship between the local cytokine production in the crevicular space and the clinical (rheumatologic and periodontal) parameters at baseline for both groups (immediate and delayed), but no significant ( $p < 0.05$ ) positive correlations were found (Table 6.4).

Additionally, correlation analysis of the changes on cytokine production and changes on clinical parameters after periodontal therapy was investigated, and only one correlation was found significant, between RANTES and ESR ( $p = 0.02, r = 0.57$ ).

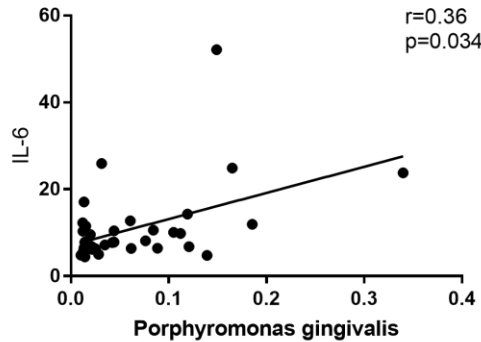
To investigate what bacteria participates in the inflammatory response of the gingival crevice in these patients, the relative abundance of the red complex bacteria (Socransky, Haffajee et al. 1998) (studied using 16s rRNA techniques, explained in chapter 5) was correlated to cytokines produced in the crevicular fluid baseline of all randomized patients that provided both GCF and plaque samples ( $n = 40$ ). Although various weak but significant associations were discovered, the strongest and most significant association found was between the relative abundance of *Cryptobacterium curtum* (CC) and TNF. CC was also correlated to a high number of pro-inflammatory cytokines including IL-8, IP-10 and MIP-1 $\alpha$ . *Porphyromonas gingivalis* (PG) was correlated with pro-inflammatory cytokines such as IL-6, TNF and IL-1 $\beta$ . *Tannerella forsythia* (TF),

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although has been described as red complex bacteria that induces pro-inflammatory cytokine production (Bodet, Chandad et al. 2006), was negatively correlated with IL-9, MIP and TNF in our study.

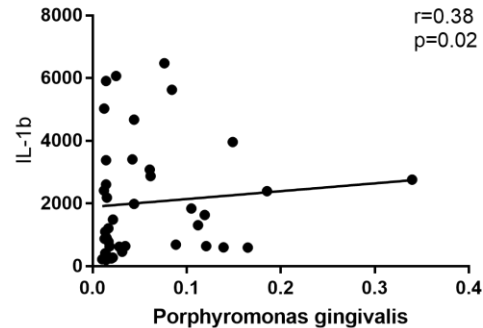
a.

Correlation analysis between relative abundance of *Porphyromonas gingivalis* and IL-6



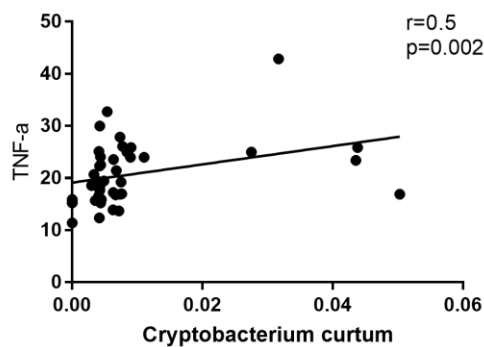
b.

Correlation analysis between relative abundance of *Porphyromonas gingivalis* and IL-1b



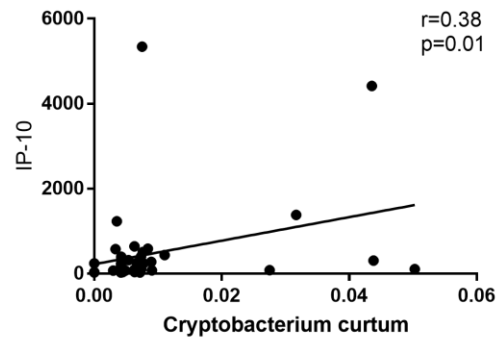
c.

Correlation analysis between relative abundance of *Cryptobacterium curtum* and TNF-alpha



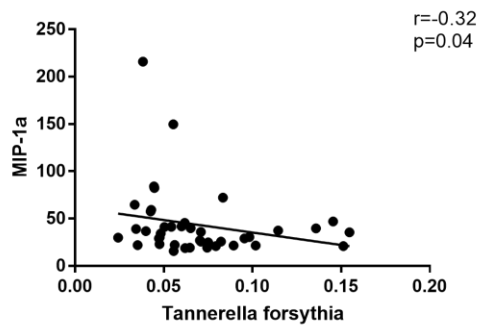
d.

Correlation analysis between relative abundance of *Cryptobacterium curtum* and IP-10



e.

Correlation analysis between relative abundance of *Tannerella forsythia* and MIP1a



f.

Correlation analysis between relative abundance of *Tannerella forsythia* and IL-9

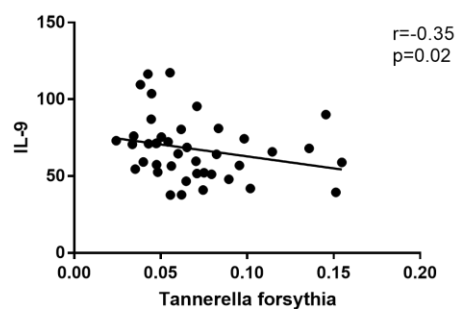


Figure 6. 7 Significant correlations between periodontal bacteria from plaque samples and cytokines from GCF samples. Test: Spearman's rank correlation, significance  $p < 0.05$ .  $N = 40$ . Abbreviations: GCF, gingival crevicular fluid; IL, interleukin; TNF, tumor necrosis factor; IP-10, inducible protein 10; MIP, macrophage inflammatory protein.

Table 6. 4 Correlation analysis between periodontal bacteria and cytokines and clinical parameters at baseline

<b>Relative abundance</b>	<b>Cytokine</b>	<b>r</b>	<b>p</b>
<b>TF</b>	IL-9	-0.35	0.026
<b>TF</b>	IL-10	-0.31	0.048
<b>TF</b>	MIP-1 $\alpha$	-0.32	0.04
<b>TF</b>	MIP-1 $\beta$	-0.36	0.02
<b>TF</b>	TNF- $\alpha$	-0.32	0.045
<b>PG</b>	IL-6	0.34	0.02
<b>PG</b>	G-CSF	0.34	0.03
<b>PG</b>	IL-1b	0.38	0.02
<b>CC</b>	IL-8	0.32	0.04
<b>CC</b>	IL-10	0.34	0.009
<b>CC</b>	IP-10	0.38	0.01
<b>CC</b>	MIP-1 $\alpha$	0.31	0.048
<b>CC</b>	TNF- $\alpha$	0.5	0.002
<b>AA</b>	IL-7	0.31	0.049
<b>AA</b>	G-CSF	0.36	0.032
<b>AA</b>	IFN-g	0.32	0.045

## 6.5. Discussion

The study of the cytokines and inflammatory mediators in RA has been crucial to understand the pathogenesis of the disease and the development of new drugs and therefore, it is important to understand the effect of periodontal therapy on these cytokines, and its association with systemic markers of inflammation. The hypothesis of this study was that, in RA patients undergoing periodontal therapy there is a reduction of local production of cytokines and that these cytokines are correlated to periodontal bacteria and markers of inflammation.

While significant differences were found in pro-inflammatory cytokines after periodontal therapy, these local cytokines were not correlated with systemic markers of inflammation at baseline of all patients included in this project. Furthermore, we did not find a correlation between the changes on cytokine production after periodontal therapy and changes on clinical parameters.

However, the local cytokine profile was correlated with some of the most important periodontal pathogens. The most significant correlation found was between *Cryptobacterium curtum* and TNF. As explained in chapter 5, this bacterium is highly associated to RA with the highest OR of all bacteria and other studies have found this bacterium in RA patients (Zhang, Zhang et al. 2015). The correlation between this bacteria and pro-inflammatory cytokines is the GCF of RA patients shows that more research is needed to elucidate what is the exact role of this bacterium in RA. Also *P. gingivalis* is significantly correlated with pro-



inflammatory cytokines such as IL-6 and IL-1b. These findings could demonstrate that periodontal pathogens orchestrate the immunological response in the periodontal pocket that can be modified with periodontal treatment. In our study, this local inflammatory response was not correlated with clinical parameters such as the DAS28 or ESR, after 6 months of periodontal therapy. Our results, do not contradict the main hypothesis, as there are limitations in this chapter such as the short follow up time, small sample size or the lack of corrections for multiple correlations (i.e. bonferroni).

Some of the most important pro-inflammatory mediators in RA are TNF, IL-1 and IL-6, which have autocrine, paracrine and endocrine effects and induce the synthesis of other cytokines, inflammatory mediators (prostaglandins, MMP, etc), acute phase response (CRP and ESR), B-cells and activation of osteoclast (Feldmann, Brennan et al. 1996). Our results show that most of these cytokines were reduced after periodontal therapy.

IL-8 is a potent chemokine produced as a response to an inflammatory stimuli that has different functions on cells of the immune system, particularly, recruiting and activating neutrophils in acute inflammatory conditions (Harada, Sekido et al. 1994) but also, in RA IL-8 is increased in synovial fluid (SF) of patients with active disease and its concentration is correlated with the number of neutrophils infiltrating the SF (Endo, Akahoshi et al. 1991). IL-9 is also a pro-inflammatory cell and anti-apoptotic, impeding the resolution of the inflammatory response, and it has been found to be significantly elevated in pre-RA patients and RA patients

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compared to controls (Kokkonen, Söderström et al. 2010). These two pro-inflammatory cytokines, in our study, experience a significant decrease ( $p=0.02$ ) after periodontal treatment in RA.

Also IL-12 and TNF were non-significantly ( $p=0.06$ ) reduced but in ACPA+ patients and in non-smokers, these reductions became significant (Figure 6.4 and 6.5). IL-12 is an interesting cytokine as has functions in the early inflammatory response against bacteria and also in the activation of the antigen-specific immunity.

VEGF (vascular endothelial growth factor) is a potent pro-inflammatory cytokine in chronic conditions and it has been described to play a key role in RA, being significantly elevated in RA patients relative to control subjects, and correlated with disease activity. Treatment of RA patients with anti-TNF significantly decreased serum VEGF, and this effect was enhanced by cotreatment with methotrexate (Paleolog, Young et al. 1998). In our study, VEGF was significantly decreased after periodontal treatment in GCF of RA patients (Figure 6.2).

MIP-1 $\alpha$  (macrophage inflammatory protein) acts as chemotractant and proinflammatory cytokine and it is in high concentrations in the gingiva of periodontitis patients (Gemmell, Carter et al. 2001). In our study, MIP- $\alpha$  was significantly decreased after periodontal treatment in GCF of RA patients.

Conversely, in the control group, none of the cytokines investigated changed significantly or close to significance. These findings might reflect that the state of

local inflammation in the gingiva of patients with chronic periodontitis is relatively stable if not treated and that, after periodontal therapy, the cytokine profile can be reverse into a healthier state.

IL-2 and IL-15 play important roles in the control of lymphocytes, as IL-2 and T-cell responses for the maintenance of self-tolerance and IL-15 as an anti-apoptotic factor (Waldmann 2015). Moreover, IL-2 and IL-15 deficient mice were associated with dysregulated proliferation of T and B-cells. In our study, we see a reduction of IL-15 and IL-2 after periodontal therapy that could be fundamental to the reduction of the inflammatory response in RA demonstrated by the significant reduction of RA parameters. Other investigators also found lower levels of IL-2 in RA synovia compared to health (Firestein, Xu et al. 1988).

In RA, synovial fluid and blood has decreased production of IL-2 compared to healthy individuals, supports the notion that T-cells are involved in pathogenesis of RA that may lead to defective T suppressor and B cell hyperactivity (Combe, Pope et al. 1985). Ebersole and colleagues found a significant increase of IL2, five months after the resolution of periodontitis (Ebersole, Kirakodu et al. 2014), which is in agreement with the results presented. Absence of IL-2 leads to susceptibility to inflammatory disorders, as there is a decline in Treg cells and increase of Th17.

It is important to mention that, in our study, some patients were on biologic drugs, which target specific molecules such as TNF (i.e. Adalimumab, infliximab) or IL-289 | Page

6 (i.e. Tolizumab) and even stops the costimulation process of T-cells (i.e. Abatacept). Although this data was available for some of the patients, the heterogeneity of the drugs used made impossible an statistical analysis in such small sample size. However, this drugs could also affect the cytokine production in the GCF.

IL-4 is the only cytokine that is increased after periodontal therapy in smokers. Interestingly, this cytokine has been shown to be reduced in health compared to RA, periodontitis and aggressive periodontitis (Havemose-Poulsen, Westergaard et al. 2006) and the absence of this cytokine enhances de expression of CD4 and macrophages and an increase of this cytokine is related to re-install the balance of inflammation and decrease of production of pro-inflammatory cytokines (Salvi, Brown et al. 1998). In smokers, this might mean that the balance of cytokines might take more than 6 months but it is initiated by IL-4 increase.

Another flaw of this chapter was that the levels of cytokines detected in GCF should have been normalized with the volume of fluid detected by the Periotron®. However, the instrument utilized for the measurement of GCF fluid was calibrated 3 years prior to finishing the study, which makes the volumes annotated unreliable (Ciantar and Caruana 1998). For this reason, these results present a semi-quantitative measurement of GCF cytokines and the units used are AU/ml instead of total amounts (pg/30 s).

## **7. CONCLUSIONS AND FINAL REMARKS**

### 7.1. Summary of main findings

The aim of this thesis was to investigate the role of periodontitis in RA and the effect of periodontal therapy on immunological and microbiological parameters. To do that, different biological samples were collected from two pilot studies, comparing RA and periodontitis patients to the appropriate controls (i.e. RA periodontally healthy, no RA periodontitis and no RA no periodontitis) and from a selected group of randomized RAPD patients before and after periodontal therapy. The specific aims were:

Objective 1: To investigate the antibody response to citrullinated and un-citrullinated peptides in sera of patients with RA and periodontitis compared to RA alone, periodontitis alone and the systemically and periodontally healthy controls and possible changes after periodontal treatment.

Objective 2: To investigate the antibody response to periodontal bacteria antigens in sera of patients with RA and periodontitis compared to RA alone, periodontitis alone and the systemically and periodontally healthy controls and possible changes after periodontal treatment.

Objective 3: To compare the subgingival microbiota of patients with RA and periodontitis, RA alone, periodontitis alone and the systemically and periodontally healthy controls and effect of periodontal therapy.

Objective 4: To investigate the differences in the cytokine response in gingival crevicular fluid in patients with periodontitis and RA (compared to controls: RA alone, periodontitis alone) and how non-surgical periodontal therapy changes immune response signatures.

To examine these objectives, antibodies to citrullinated proteins and bacterial-related antigens were quantified in serum using ELISA methods, local inflammatory markers were measured from GCF using a multiplex assay of 27 cytokines and plaque microbiome was identified using 16S rRNA analysis using the Illumina MiSeq platform.

The findings from the study of the antibodies to citrullinated and uncitrullinated human proteins in serum showed no significant difference between the RA group with or without periodontitis and the systemically healthy patients with and without periodontitis (Chapter 3). Although a slight elevation of antibodies was observed in the periodontitis groups, it could not be confirmed that periodontitis produces an elevation of antibodies to citrullinated proteins. In the longitudinal analysis, although periodontal therapy significantly reduced RA and periodontal clinical parameters, there was no effect on the level of antibodies against citrullinated proteins.

The findings from the study of antibodies to bacterial proteins from serum showed that RA patients have an increased production of antibodies against periodontal bacteria, in both periodontally healthy and periodontitis groups (Chapter 4).

Although the number of patients with antibody positivity was low, these results suggest that *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and PPAD but not RgpB or *Prevotella intermedia*, act like an antigen in RA provoking an antibody reaction that does not appear to be due to ACPA cross reactivity. However, periodontal therapy did not reduce the production of antibodies to PG-antigens or FN after 6 months.

The findings from the microbiome analysis of RA patients showed that both RA and PD (and not age, gender or other clinical parameters) shift the oral microbiome, facilitating the growth of anaerobic bacteria (Chapter 5). Interestingly, even in periodontally healthy patients, RA leads to a more anaerobic and Gram-negative oral microbiome, with species such as *Cryptobacterium curtum* being highly elevated (100-fold greater abundance) in RA. While PD also have a major role in shaping the oral microbiome in RA and systemically healthy patients, periodontal therapy only affected the bacterial composition after 3 months but not after 6 months, following the same pattern that the clinical parameters. This reinforces the importance of periodontal maintenance and continuous treatment to reach periodontal health.

The findings from the study of inflammatory cytokines before and after periodontal therapy in RA showed a significant reduction of pro-inflammatory cytokines after periodontal therapy (Chapter 6). Cytokines such as IL-8, IL-9, VEGF and MIP-1a were significantly reduced 6 months after periodontal therapy, and others such as IL-10, IL-12 and TNF were reduced with p values lower than



0.6 (close to significance). Considering the small sample size, it can be concluded that in RA, periodontal therapy lead to a reduction of local pro-inflammatory cytokines that did not occur in the control group, significantly and positively correlated with the relative abundance of some periodontal bacteria, such as *P. gingivalis* and *Cryptobacterium curtum*.

## 7.2. Limitations

The study design used in this thesis had numerous flaws, which makes difficult the interpretation of the results presented in this thesis. As a pilot study, the OPERA trial aimed to collect feasibility information and was not powered or designed for the realization of the aims of this thesis. For that reason, certain limitations such as the number of patients or certain methodologies need to be considered when assessing the relevance of the results presented here.

The inclusion criteria for the OPERA study aimed to ensure a minimum of RA and periodontal disease activity. However, we believe that both the RA criteria (minimum of 3.2 DAS28) and periodontal criteria (minimum of 40 mm of cumulative probing depth) represent in some cases a mild disease spectrum. Having a mild disease group impedes finding differences with the healthy control, which could explain the results found in Chapter 3 and 4. Also, having low disease activity impedes finding a significant effect of periodontal therapy in the longitudinal part of the study, as although both RA and PD parameters were reduced in all chapters in the control group, the differences are rather small.

Measuring the effect of any treatment in RA patients is challenging due to the nature of RA. If patients are not in remission, this chronic disease is characterized by numerous immunological changes due to flare ups and changes in medications. These changes could be silencing the effect of periodontal therapy and adds to the great patient variability observed in this thesis.

The results of this thesis support that *P. gingivalis* remains a candidate for the trigger of autoimmune response in RA. While antibodies to *P. gingivalis* were elevated in RA, the presence and abundance of PG in plaque was not correlated with antibodies against *P. gingivalis*, which indicates that there seem to be a specific immunological response to this periodontal bacterium in RA, not due to an increase of *P. gingivalis* in periodontitis but because it is recognized as an antigen. This specific antigen recognition could therefore be the trigger for the antibody reaction in RA but more research is needed in longitudinal trials with pre-RA patients. While it has been discussed that this elevation of antibodies against *P. gingivalis* could represent a cross-reaction with antibodies to citrullinated proteins, the elevation of antibodies to non-citrullinated proteins from *P. gingivalis* (such as RPP3, PG-eno and OMA-PG) shows this is unlikely but to truly verify this, a cross-reactivity experiment should be conducted, so our hypothesis cannot be confirmed.

The methodology used in each chapter also revealed some limitations. In the antibody analysis, no standard curve was available for some of the antigens, such as RPP3 and a low concentration of antibodies was found in all subjects. Thus,

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the results from those experiments are not as robust and conclusions should be made cautiously. For the analysis of the cytokine array, the data should have been normalized using the volume of GCF collected in 30 seconds measured with the Periotron®. Unfortunately, as this instrument was not appropriately calibrated, cytokine concentration was presented in AU/ml instead of total amounts (pg/30 s), being a semi-quantitative method. Also, the microbiome analysis using 16S phylogenetic marker is considered the gold standard, this method has limitations, such as the production of PCR artifacts, and the relatively low resolution at species level (Pontes, Lima-Bittencourt et al. 2007). Also, using 16S sequencing does not provide information about functions or sub-species of bacteria, which can be obtained through other methods such as whole-metagenome shotgun sequencing, which studies the entire genetic microbial composition of an environment.

The small sample size in the longitudinal groups amplifies the impact of the patient variability found in our results. Although all experiments were conducted using biological samples collected from the same randomized group of patients, when evaluating the effect of periodontal therapy in DAS28 or ESR levels, a change of a few patients due to drop outs or due to inexistence of certain sample, changes the significance. This loss of significance is due to the small group of patients and high heterogeneity and a larger group needs to be investigated to overcome this limitations.

Effectiveness of one single course of periodontal therapy has been previously discussed and some periodontologists insist that continuous treatment has to be offered or the clinical and microbiological signs of periodontitis return after 2 months (Sbordone, Ramaglia et al. 1990). As this periodontal maintenance was not provided in our study and plaque scores were not recorded, the effect of periodontal therapy in the microbiome or immune system after 6 months is expected to be low, which can explain our negative results.

### 7.3. Conclusions and future research

The findings from this thesis lend further credence to the link between RA and the oral microbiome, with RA patients having a disrupted and more anaerobic microbiota and an exacerbated immunological reaction against periodontal bacteria and citrullinated proteins. However, the directionality or causality cannot be explored with this cross-sectional study design. Longitudinal studies that follow up RA and PD patients before and after they are diagnosed, are necessary to identify the directionality and the driver species (such as *Cryptobacterium curtum*) that could serve as markers for RA diagnosis and stratification of patients and to assess if oral dysbiosis occurs as a pre-clinical phase of periodontitis, RA, both or none. The results presented in the longitudinal study sets the basis to follow for a larger and controlled randomized controlled trial that, by increasing the sample size and correcting the errors from our protocol, overcomes the limitation of studying such variable disease.

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## **8. Appendices**

### 8.1. Calibration curves for antibody ELISAs

Preliminary experiments were necessary to find appropriate positive controls to use in the plates that tested the antibody levels against ACAPAs and PG-antigens. To that end, patient sera was diluted following a standard curve and plates were tested (Figure 8. 1, Figure 8. 2, Figure 8. 3, Figure 8. 4). However, no appropriate control was found for the arginated peptides (non-citrullinated) or RPP3 and PPAD, due to the lack of patients with strong antibody reaction against this antigens.

These results demonstrate low antibody reaction to the arginated human proteins in this study and limits the reliability of the results found from the non-controlled antigens tested.

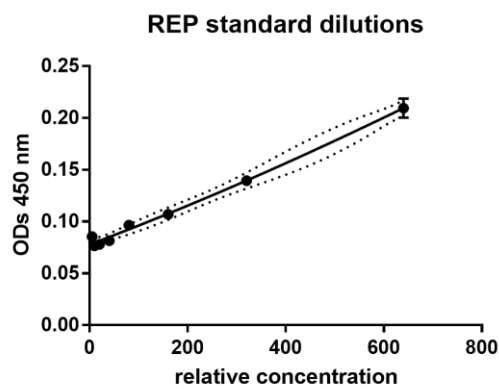
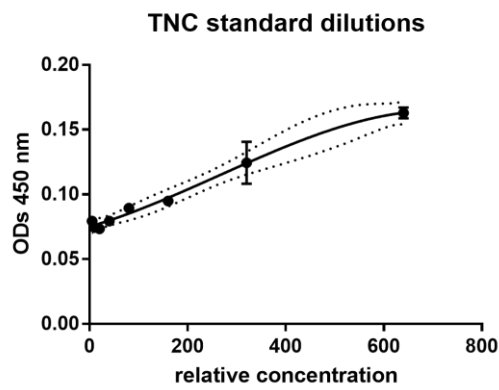
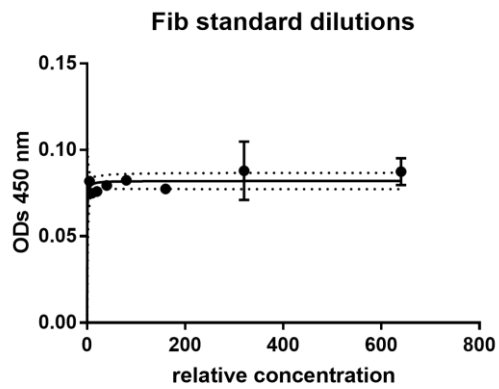


Figure 8. 1 Failed attempts to find calibration curves for antibody ELISAs to human antigens. Methods explained in section 2.2.3. Optical densities (ODs) are measured at 450 nm and data is represented as relative concentrations of diluted standards.

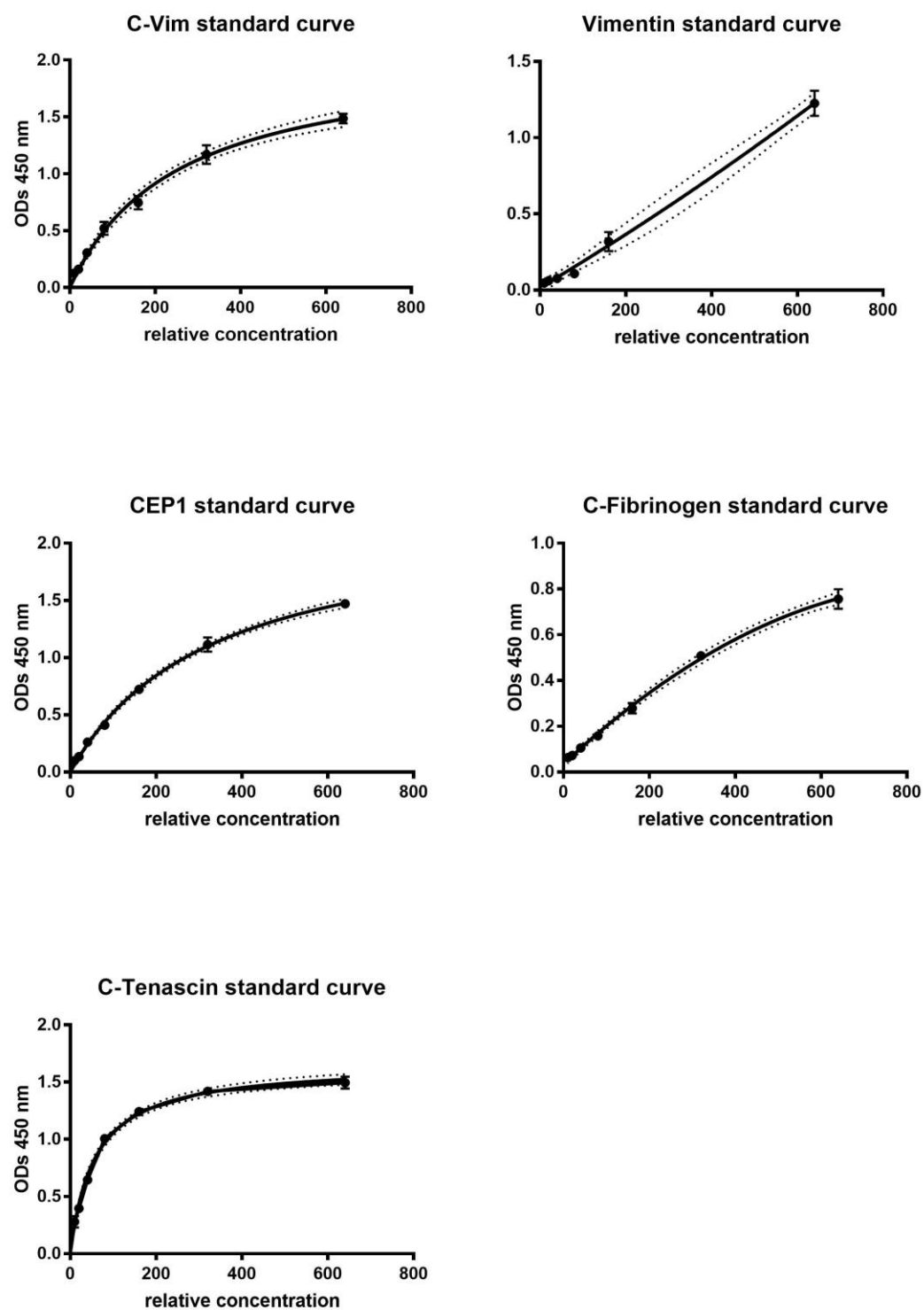


Figure 8. 2 Calibration curves for antibody ELISAs to human antigens. Methods explained in section 2.2.3. Optical densities (ODs) are measured at 450 nm and data is represented as relative concentrations of diluted standards.



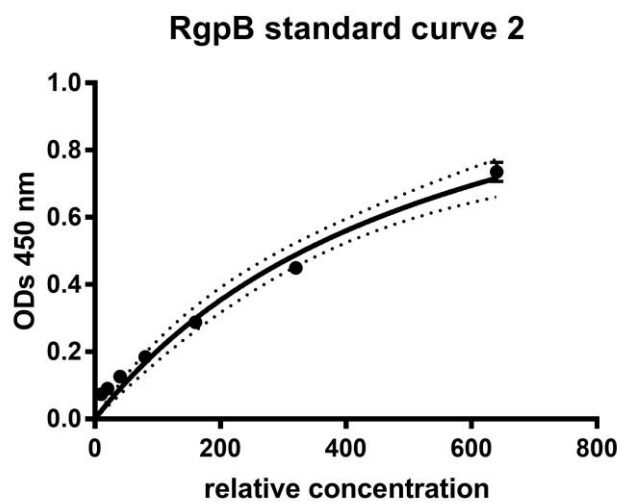
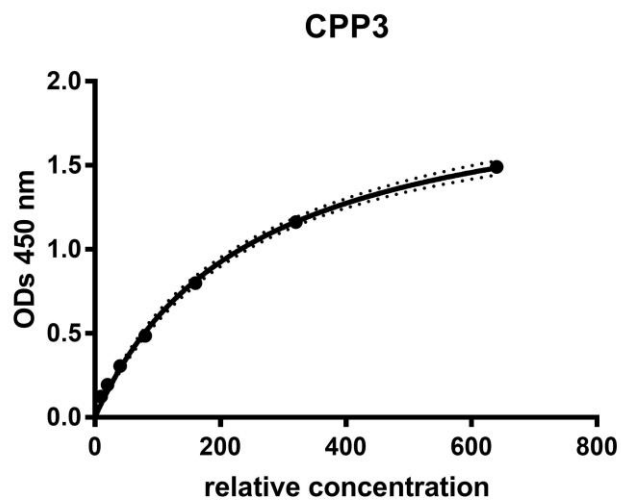


Figure 8. 3 Calibration curves for antibody ELISAs to PG-related antigens. Methods explained in section 2.2.3. Optical densities (ODs) are measured at 450 nm and data is represented as relative concentrations of diluted standards.

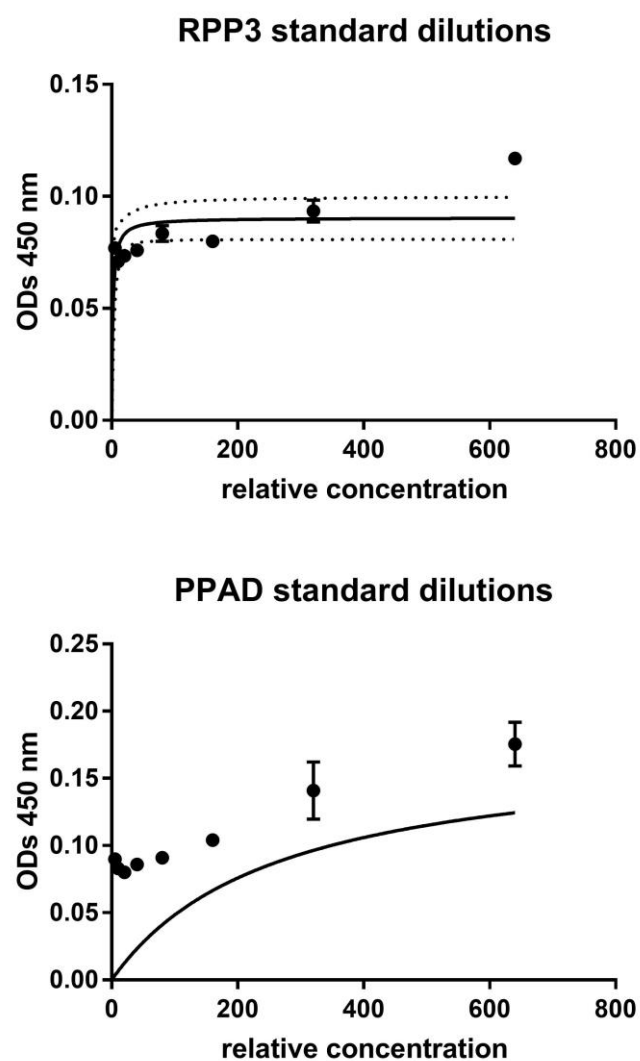


Figure 8. 4 Failed attempts to find calibration curves for antibody ELISAs to PG-related antigens. Methods explained in section 2.2.3. Optical densities (ODs) are measured at 450 nm and data is represented as relative concentrations of diluted standards.

## 8.2. Correlation analysis between circulating antibodies and clinical parameters

Weak significant correlations were found between the antibody production against proteins from PG, FN and PI which suggests that these bacteria, in agreement with the current literature, are positively and significantly correlated (Table 8. 1). Clinical parameters related to periodontitis are strongly and significantly associated with RA parameters.

However, although some of these significant correlations are strong and others are weak, the small sample size limits the reliability of the results. Although this study suggests a correlation between circulating antibodies to periodontal bacteria and clinical parameters in RA, a pivotal study with a bigger sample size is needed to verify this results.

Table 8. 1 Significant correlations of crosssectional variables at baseline (Spearman's correlation,  $p < 0.04$ ).

Variable 1	Variable 2	r	p
anti-PG	C-TNC5	0.287	0.02
anti-PG	RgpB	0.359	0.001
anti-PG	CCP	0.215	0.023
anti-PI	anti-PG	0.2	0.02
anti-PI	anti-FN	0.19	0.03
anti-PI	PPAD	-0.2	0.02
anti-FN	anti-PG	0.39	0.001
anti-FN	PG-eno	0.4	0.001
anti-FN	PPAD	0.3	0.01
anti-PI	DAS28	-0.197	0.034
anti-PI	ESR	-0.226	0.015
anti-PI	Age	-0.394	0.001
anti-PG	cumPPD	0.288	0.002
anti-PG	Cal	0.32	0.001
anti-PG	BoP	0.217	0.019
anti-FN	BoP	0.193	0.039
anti-FN	PPD	0.276	0.003

anti-FN	DAS28	0.273	0.01
anti-FN	ESR	0.239	0.01
PPD	DAS28	0.81	0.001
PPD	ESR	0.77	0.001
CAL	DAS28	0.344	0.001
CAL	ESR	0.32	0.001

### 8.3. Conference abstracts

Circulating Antibodies to Citrullinated Proteins in Rheumatoid Arthritis and Periodontitis, I. Lopez-Oliva, A. Montgomery, A. Quirke, P. Venables, T.Dietrich, S. Serban, P. de Pablo, K.Raza, A. Filer, M. M. Grant. (J Dent Res 96 #(Spec Iss A): 2629694, 2017)

Outcomes of periodontal therapy in Rheumatoid Arthritis: baseline data from a randomised controlled trial Lopez-oliva, I. , Stefan, S. , de Pablo, P. , Filer, A. , Raza, K. , Dietrich, T. BSODR 2015.

The oral microbiome in periodontally healthy rheumatoid arthritis patients, I. Lopez-Oliva, A. Paropkari, T.Dietrich, S. Serban, P. de Pablo, K.Raza, A. Filer, M. M. Grant, P. Kumar. Abstract number: 2768542, BSODR-IADR17/ British Society for Oral and Dental Research.

#### 8.4. Awards, grants and prizes

College of Medical and Dental Sciences Postgraduate Researcher Overseas Work Experience Scholarship (£3,000) to attend Dr Purnima Kumar microbiomics laboratory in Ohio State (May-June 2016)

School travel fund (£500) to attend Dr Patrick Venable laboratory in Oxford University for 2 months (March-April 2016)

GLAXO SMITH KLINE / ODRT grant, (£6,495) for laboratory experiments and to attend Purnima Kumar microbiomics laboratory in Ohio State (November-December 2016).

Past Presidencies IADR travel award (3,000\$) for best research paper at the IADR 2017 annual conference in San Francisco (USA).

Last round Finalist for the 3 Minute Thesis competition 2017 (University of Birmingham, UK).

Last round Finalist for the Sir Wilfred Fish Prize 2017 (British Society of Periodontology, London, UK).

### 8.1. Publications

Periodontitis and Rheumatoid Arthritis, Isabel Lopez-oliva, Paola de Pablo, Iain Chapple, Thomas Dietrich, Melissa Grant. Periodoncia clinica 2017, Vol. 8.

The oral microbiome in periodontally healthy rheumatoid arthritis patients, I. Lopez-Oliva, A. Paropkari, T.Dietrich, S. Serban, P. de Pablo, K.Raza, A. Filer, M. M. Grant, P. Kumar. Accepted for Arhtritis & Rheumatology journal

























**Title:** Dysbiotic subgingival microbial communities in periodontally healthy patients with rheumatoid arthritis

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**KEY WORDS:** Rheumatoid arthritis, periodontitis, DNA Sequence Analysis, oral microbiome

**Word count:** 1,493

## Abstract

### Objectives

Studies that demonstrate an association between rheumatoid arthritis (RA) and dysbiotic oral microbiomes are often confounded by the presence of extensive periodontitis in these individuals. Therefore, the present investigation sought to investigate the role of RA in modulating the periodontal microbiome by comparing periodontally healthy individuals with and without RA.

### Methods

Subgingival plaque was collected from was collected periodontally healthy individuals (22 with and 19 without RA), and 16S gene sequenced on the Illumina MiSeq platform. Bacterial biodiversity and co-occurrence patterns were examined using the QIIME and PhyloToAST pipelines.

### Results

The subgingival microbiomes differed significantly based on both community membership and as well as the abundance of lineages, with 41.9% of the community differing in abundance and 19% in membership. In contrast to the sparse and predominantly congeneric co-occurrence networks seen in controls, RA subjects revealed a highly connected grid containing a large inter-generic hub anchored by known periodontal pathogens. Predictive metagenomic analysis (PICRUSt) demonstrated that arachidonic acid and ester lipid metabolism pathways might partly explain the robustness of this clustering. As expected from a periodontally healthy cohort, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were not significantly different between groups, however, *Cryptobacterium curtum*, another organism capable of producing large amounts of citrulline, emerged as a robust discriminant of the microbiome in individuals with RA.

### Conclusions

Our data demonstrates that the oral microbiome in RA is enriched for inflammophilic and citrulline producing organisms, which may play a role in the production of autoantigenic citrullinated peptides in RA.

## INTRODUCTION

Rheumatoid arthritis (RA) has been associated with periodontal disease (PD), a bacterially initiated chronic inflammation that leads to destruction of tooth-supporting tissues(Kaur, White et al. 2012). Although PD and RA share similar inflammatory pathways as well as genetic and environmental risk factors, these are insufficient to explain this connection(Kaur, White et al. 2012).

While the cause of RA remains unknown, it has been hypothesized that oral microbiota(de Pablo, Chapple et al. 2009, de Pablo, Dietrich et al. 2014) in particular the periodontal pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*, may play a critical role in its pathogenesis(Wegner, Wait et al. 2010, Konig, Abusleme et al. 2016)

Studies using next generation sequencing methods demonstrate the oral microbiome is altered in RA(Scher, Ubeda et al. 2012, Zhang, Zhang et al. 2015). However, the majority of these studies included individuals with moderate to severe periodontitis(Scher, Ubeda et al. 2012) or individuals whose periodontal health status was not established(Zhang, Zhang et al. 2015). Periodontitis, by itself, is a significant modifier of the oral microbiome(Griffen, Beall et al. 2012), making it difficult to dissect the relative contributions of periodontitis and RA to the microbial dysbiosis.

Given the potential role of oral bacteria may play in the etiopathogenesis of RA, we set out to characterize the periodontal microbiome in periodontally healthy individuals with and without RA, using next generation sequencing.

## METHODS

The study sample included patients with RA and non-RA controls. All participants were periodontally healthy. Subgingival plaque samples were collected and analyzed using 16S rDNA sequencing. Detailed methods are described in supplementary information. The sequences are deposited in the Sequence Read Archive of NCBI (project number: PRJNA391575).

## RESULTS

We examined 22 patients with RA and 19 non-RA controls. There was a statistically significant but clinically inconsequential difference between groups in periodontal measures, in particular PPD and CAL (Table 1). Principal Coordinate Analysis (PCoA) of both unweighted and weighted UniFrac distances demonstrated significant clustering of the microbiomes based on RA status (Figure 1,  $p=0.001$ , Adonis test), indicating that these groups differed both in presence or absence of lineages (community membership), as well as in the relative abundances of lineages within communities (community structure).

Table 1: Clinical and demographic characteristics in periodontally healthy subjects with rheumatoid arthritis (RA) and without RA (non-RA). Data represented as mean (25, 75 percentile) for ordinal data and percentage for categorical data. P values are calculated using Mann-Whitney test for ordinal data and Fisher's test for categorical data and significant differences ( $p < 0.05$ ) indicated with an asterisk (\*). Abbreviations: BMI, body mass index; PPD probing pocket depth; BoP, bleeding on probing; CAL, clinical attachment loss; ESR, erythrocyte sedimentation rate; VAS, visual analogue scale for patients global assessment of disease activity; DAS, disease activity score

	RA n=22	Non-RA n=19
Age in years, mean (IQR)	60 (54.1, 63.4)*	36 (32.9, 41.6)
Gender (% Male)	23	32
Ethnicity (%)		
White	95	89
Asian	5	11
Smoking history (%)		
Never	62	90
Former	29	5
Current	9	5
Alcohol consumption (%)		
Never	11	14
1-4 times/month	73	45
1-4 times/week	16	41
Clinical periodontal characteristics		
PPD in mm, mean (IQR)	2.3 (2.2, 2.4)*	1.6 (1.5, 1.7)
Number of sites with PPD>4mm	1.2 (0, 2)	0.9 (0,3)
Number of sites with BoP, mean (IQR)	6 (0, 19)	4 (1, 16)
Gingival recession in mm, mean (IQR)	0.28 (0.01, 0.26)*	0.13 (0.04, 0.2)
Measures of RA severity		
ESR	8 (8.7, 21.7)	
VAS (global assessment of disease activity)	41 (31.7, 58.5)	
DAS28	3.4 (2.7, 3.9)	

Since patients with RA differed from controls in both community membership and structure, we identified species level operational taxonomic units (s-OTUs) that contributed to this difference using an increasingly granular top-down approach.

Patients with RA presented had greater abundances of obligate anaerobes (both gram-positive and gram-negative) while facultatives (especially gram-negative) were identified in greater abundance in non-RA controls ( $p < 0.05$  Wilcoxon signed rank test, Figure 1).

We then used DESeq2 (Love, Huber et al. 2014) to identify differentially abundant OTUs; with  $p$ -values  $< 0.05$  after adjusting for multiple testing, and Fisher's exact test to examine the frequency of detection. We identified 558 OTUs from 3,963,291 classifiable sequences (mean of 107115 sequences per sample, range 69626-182993). Rarefaction curves demonstrated that all samples approached saturation or had plateaued. 229 OTUs (41.9% of the community) differed significantly in structure and 105 OTUs (19%) differed significantly in membership between groups (Figure 1 and supplementary table 1). Certain species were significantly more abundant in patients with RA, including those belonging to the genera *Actinomyces* (odds ratios (OR) varying from 4-9 for each species within the genus), *Cryptobacterium* (OR=36), *Dialister*, *Desulfovibrio* (ORs of 4 and 26), *Fretibacterium* (OR 9 to 12), *Leptotrichia* (OR 7 to 26) *Prevotella* (OR 0.04 to 6), *Selenomonas* (OR 0 to 7), *Treponema* (OR 0 to 7), and *Veillonellaceae* [G1] (OR 0 to 6).

In contrast, several species belonging to the genera *Aggregatibacter*, *Gemella*, *Granulicatella*, *Hemophilus*, *Neisseria* and *Streptococci* not only demonstrated lower abundances but also were less frequently detected in RA. These significantly abundant species accounted for a median of 28% (range 12-82%) of each individual's microbiome in patients with RA, indicating that these differences are not attributable to the rare biosphere.

Since the subgingival microbiome is known to be significantly heterogeneous among individuals (Paster, Boches et al. 2001), we used the core microbiome (suite of species identified in  $\geq 80\%$  of subjects) to compare stable associations between groups. 326 OTUs were identified in the core microbiome of all study participants and 364 in patients with RA. 27.7% of the community (101 OTUs) differed significantly in structure and 10.9% (40 OTUs) in membership, with 38 species unique to the RA core microbiome (Figure 1). Importantly, 157 of the 229 species identified above belonged to the core microbiome.

Sparse, congeneric networks were observed in non-RA controls (Figure 2). On the other hand, the network topology of individuals with RA revealed a highly connected grid with a robust intergeneric hub. 83 of the 157 core species were incorporated in this hub, further reinforcing our observation that in subjects with RA, the environment imposes a selection drive. Importantly, known pathogenic species belonging to *Treponema*, *Selenomonas*, *Filifactor*, *Campylobacter* and *Fretibacterium* were tightly interwoven into this hub, and 12 gram-negative species were identified as network anchors. Interestingly, species traditionally associated with RA, for example, *P.gingivalis* (Pg) and *A.actinomycetemcomitans* (Aa), were not part of the network cluster.

Since there is little literature-based information to provide insights into the biological basis for this tight clustering, we combined predictive metagenomic analysis

(PICRUSt(Langille, Zaneveld et al. 2013)) with network graph theory and core microbiome analysis to explore if shared functionality could explain co-occurrence (Figure 2). Bacterial arachidonic acid and ether lipid metabolism genes exhibited the greatest betweenness centrality (reflecting the amount of control that these node exerts over the interactions of other nodes in the network(Yoon, Blumer et al. 2006)), and the highest degree centrality (an indication that they are the central focal point of the structure).

## DISCUSSION

Gram-negative anaerobes are known to play important roles in initiating periodontitis, and emerging evidence also implicates them in the etiopathogenesis of RA(Mikuls, Payne et al. 2014, Zhang, Zhang et al. 2015). Our results show that even in periodontally healthy RA patients, gram-negative anaerobes are significantly more abundant in RA, consistent with a dysbiotic state. Such a status might indicate a pre-clinical phase of periodontitis. As expected from a periodontally healthy adult cohort, *Pg* and *Aa* were neither dominant members of the microbiome nor significantly different between groups. Taken together with previous studies(Mikuls, Payne et al. 2014), our data implies that gram-negative bacteria other than *Pg* and *Aa* may play a role in initiation of RA, while the evidence from literature suggests that these two species may be critical to disease perpetuation.

Recent investigations demonstrate that while substantial microbial heterogeneity exists among healthy individuals, a robust core microbiome is identifiable in individuals who smoke or are pregnant(Mason, Preshaw et al. 2015, Jetté, Dill-McFarland et al. 2016, Paropkari, Leblebicioglu et al. 2016). The findings of the present study parallel these previous observations and support the ecological plaque hypothesis(Marsh 1994), suggesting that RA imposes a habitat filtering on the subgingival environment, preferentially promoting the growth of certain organisms.

Traditional statistical methods assume bacterial presence and abundance to be independent variables, but in reality bacterial presence in a biofilm is driven by inter-dependent nutritional and metabolic interactions. Therefore, we combined network graph theory with DESeq and core microbiome analysis to examine co-occurrence patterns and identify important community members (network anchors). No network anchors were identifiable in controls (since betweenness centrality was homogeneous between species), indicating that this is an ecological niche in equilibrium. However, the tightly woven hub of anaerobes suggest that a small group of anaerobic bacteria play an important role controlling the flow of resources in the RA-influenced microbiome, implying that even small changes in these anchors could impact upon community assembly in people with RA. These species may be potential targets for microbial disruption.

Arachidonic acid (AA) is essential for cell membrane integrity. It is metabolized to prostaglandin E2 (PGE<sub>2</sub>) and other pro-inflammatory eicosanoids, which are implicated in the development of RA. The ability to metabolize AA into pro-inflammatory eicosanoids



is an emergent property of opportunistic pathogens(Fourie, Ells et al. 2016). AA is also known to inhibit the growth and epithelial adhesion of beneficial species in the gut(Kankaanpaa, Yang et al. 2004). Taken together, the data indicate that the subgingival microbiome is both influenced by, and influences, the inflammatory burden of RA.

One of the most intriguing findings was the identification of *Cryptobacterium curtum* as a predominant member of the RA-influenced periodontal microbiome. This gram-positive, assacharolytic, anaerobic rod (which was previously misclassified as *Eubacterium saburreum*) degrades arginine through the arginine deiminase pathway and produces substantial amounts of citrulline, ornithine and ammonia(Uematsu, Sato et al. 2006). We have previously identified this as a periodontal pathogen(Kumar, Griffen et al. 2003), and translocation from oral sources has been implicated in the etiology of distant infections such as pelvic abscesses, gynecologic infections, and wounds(Brook and Frazier 1993). More importantly, *C. curtum* is enriched in the oral and gut microbiomes of early RA cases(Vahtovuori, Munukka et al. 2008, Zhang, Zhang et al. 2015). In line with previous studies, we observed that this species was a member of the core microbiome in RA patients. Compared to non-RA controls, this species demonstrated a 100-fold greater abundance in RA with 39-fold greater odds of detection. While this unusually high association does not necessarily suggest an etiopathogenic role for *C. curtum*, this organism is certainly a candidate for further studies. In light of evidence that antibodies against citrullinated protein and peptides (ACPA) precede the clinical onset of RA by several years, have high specificity for RA at over 95%(Schellekens, de Jong et al. 1998, Schellekens, Visser et al. 2000) and that we previously observed antibodies characteristic of RA, including citrullinated and uncitrullinated peptides of the RA autoantigens in individuals with periodontitis(de Pablo, Dietrich et al. 2014), the ability of *C. curtum* to degrade arginine via the arginine deiminase pathway and to produce substantial amounts of citrulline is of particular interest. Presence of *C. curtum* in the plaque may therefore be a contributing factor in the development of RA autoantigens and warrants further investigation.

In summary, our data suggest that RA plays a major role in shaping the oral microbiome. The microbiome in RA is enriched for pro-inflammatory organisms and those capable of producing substantial amounts of citrulline (pro-antigenic). An ability to metabolize arachidonic acid and ether lipids appears to be a shared function among the species observed in individuals with RA. Our findings lend further credence to a link between the oral microbiome and RA; however, longitudinal studies are needed to understand directionality and causality, and also to characterize potentially "driver species" that could serve as biomarkers for RA.

## TABLES AND FIGURES

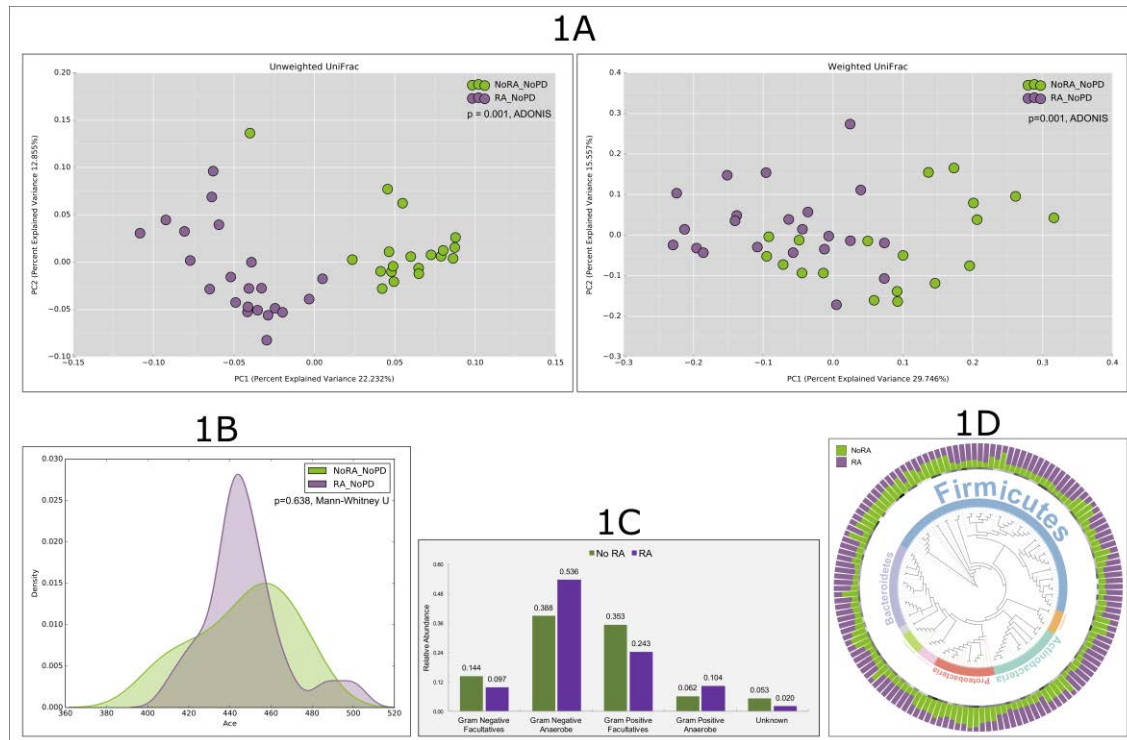


Figure 1: Differences in alpha and beta diversity metrics between periodontally healthy subjects with and without rheumatoid arthritis (RA). (A): Principal Coordinates Analysis (PCoA) plots of unweighted and weighted Unifrac distances (B): Kernel plots of alpha diversity (Abundance-based Coverage Estimator (ACE)). The peak indicates the median values for each group. The x-axis indicates the data range. (C): Distribution of species by gram staining and oxygen requirement characteristics. Groups that share the same symbol are significantly different from each other ( $p < 0.05$ , Kruskal Wallis test) (D): Phylogenetic tree representing normalized mean relative abundance (NMRA, stacked bar chart), core species (circles represent species present in  $\geq 80\%$  of samples in a group), significant frequency of detection (stars) and phylum-level taxonomic annotation (colored-strips and text) for significantly different and differentially abundant species-level OTUs (tree leaves). Data for figure 1D is presented in supplemental table 1.

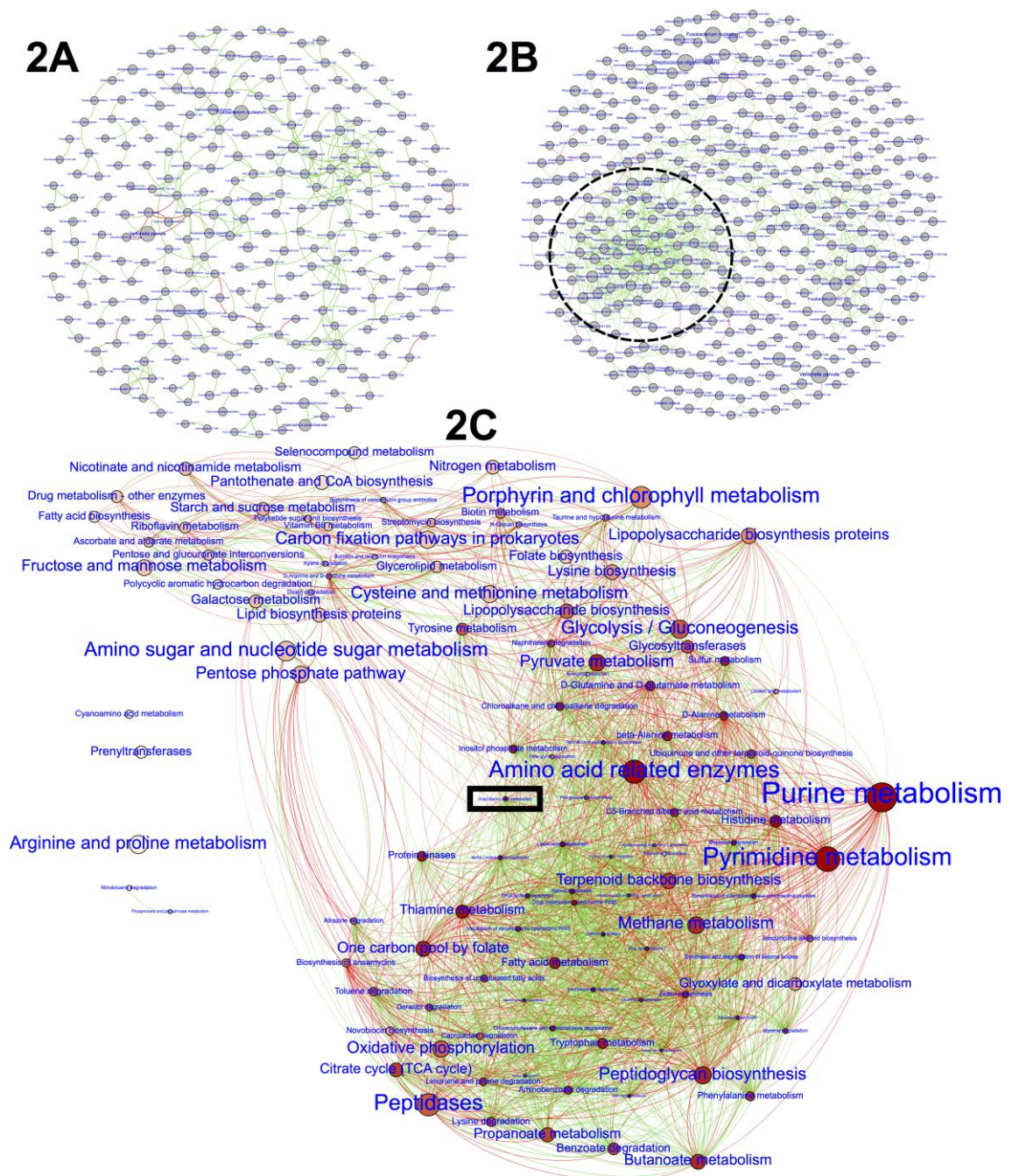


Figure 2: Co-occurrence networks in periodontally healthy subjects with or without rheumatoid arthritis (RA): Each network graph contains nodes (circles) and edges (connections representing Spearman's  $\rho$ ). Edges are colored green for positive correlation and red for negative correlation. Nodes represent species-level OTUs in 2A and 2B and genes encoding for metabolic functions in 2C; and are sized by relative abundance. Edges represent significant and robust Spearman's correlation ( $p < 0.05$ ,  $\rho \geq 0.75$ ). Data for figure 2C is presented in supplemental table 2.

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